Immune reconstitution in cord blood transplantation

Vikesh Devlia

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Anthony Nolan Research Institute
Cancer Institute
University College London (UCL)

Declaration

I, Vikesh Devlia, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature……

Printed Name: Vikesh Devlia

Date……..

Abstract

Umbilical cord blood (UCB) is a readily available option as a stem cell source for those requiring an allogeneic transplant as part of curative therapy for malignant or non-malignant haematological disorders. Immune reconstitution is essential in cord blood transplant (CBT) patients to reduce the development of post-transplant complications. The kinetics and diversity of reconstituting immune cells has not been comprehensively investigated in CBT patients within the UK. Thus, in this study the kinetics and diversity of reconstituting immune cell subsets within CBT patients in the UK has been analysed.

CBT patients provided whole blood samples at 28, 60, 100, 180, 365 and 720 days post-CBT. Flow cytometry was used to determine the kinetics and diversity of reconstituting immune cells. T-cell receptor excision circles (TRECs) and kappa deleting recombination excision circles (KRECs) were quantified using Real Time PCR (RT-PCR) to measure thymic and bone marrow output. NK cell function was determined through immunophenotyping of stimulatory and activating markers and cytotoxicity directed to K562 cells.

CD45+ cells and CD14+ monocytes reconstitute by 60 days post-CBT. CD19+ B cells reconstitute by 100 days and a higher absolute count of CD19+ B cells at 28 days post-CBT correlates with improved overall survival. T cell reconstitution is delayed for up to 720 days for CD3+/CD4+ T cells and 365 days for CD8+ T cells. Thymic output is delayed for up to 720 days with low absolute levels of naïve T cells and low TREC copy numbers. However, increased numbers of effector memory T cells demonstrate thymic independent expansion of T cells. NK cells are activated, producing IFN-γ and mount a cytotoxic response towards K562 cells. Therefore, this project provides an insight into the kinetics and diversity of reconstituting immune cell subsets in CBT patients, serving as a timeline that can be used clinically.

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Table of Contents

Tables

Figures

Abbreviations

A	Adenine
ADCC	Antibody-dependent cell-mediated Cytotoxicity
ADV	Adenovirus
aGvHD	acute graft versus host disease
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cells
ATG	Anti-Thymocyte Globulin
β2m	Beta-2-Microglobulin
β-ME	Beta-mercaptoethanol
BM	Bone marrow
BMT	Bone marrow transplantation
bp	base pairs
BSA	Bovine serum albumin
Bu	Busulfan
C	Cytosine
CB	Cord blood
CBB	Cord blood bank
CBMCs	Cord blood mononuclear cells
CBT	Cord blood transplantation
cGvHD	chronic graft versus host disease
CLL	Chronic lymphocytic leukaemia
CLP	Common lymphoid progenitor
CLIP	Class II-associated invariant chain peptide
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CsA	Ciclosporin A
CTL	Cytotoxic T lymphocyte
CY	Cyclophosphamide
dCBU	double cord blood unit
DCs	Dendritic cells

DLI	Donor lymphocyte infusion
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
dUCBT	double umbilical cord blood transplant
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EtBr	Ethidium Bromide
FCS	Fetal calf serum
FCγR	Fcγ receptors
Flu	Fludarabine
G	Guanine
G-CSF	Granulocyte colony-stimulating factor
gDNA	genomic deoxyribonucleic acid
GI	Gastrointestinal
GvHD	Graft versus host disease
GvL	Graft versus leukaemia
Gy	Grays
h	hour
HLA	Human leukocyte antigen
HPE	Homeostatic peripheral expansion
HSC	Haematopoietic stem cells
HSCT	Haematopoietic stem cell transplantation
IGC	Immunoglobulin constant gene
IFN-y	Interferon-gamma
IGK	Immunoglobulin kappa locus
iTregs	inducible regulatory T cells
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
kDa	kilodalton
KRECs	Kappa deleting recombination excision circles
Lck	lymphocyte-specific protein tyrosine kinase

LT-HSC	Long term haematopoietic stem cell
MA	monoclonal antibody
MAC	myeloablative conditioning
Mel	Melphalan
MFI	Mean of fluorescence intensity
MgCl2	Magnesium chloride
MHC	Major histocompatibility complex
mins	minutes
MMF	Mycofenolate mofetil
mRNA	Messenger ribonucleic acid
MRD	matched related donor
mPB	mobilised peripheral blood
MUD	Matched unrelated donor
NaCl	Sodium chloride
NFAT	Nuclear factor of activated T cells
NK	Natural Killer
NKT	Natural Killer T cell
NMA	Non myeloablative conditioning
PAMP	Pathogen associated molecular patterns
PB	Peripheral Blood
PBMC	Peripheral Blood mononuclear cells
PCR	Polymerase chain reaction
Pen/strep	penicillin and streptomycin
PRR	Pattern recognition receptors
RIC	Reduced intensity conditioning
RPMI	Roswell Park Memorial Institute Media with L-glutamine
RSS	Recombination signal sequence
RTE	Recent thymic emigrant
sCBU	single cord blood unit
ST-HSC	short-term haematopoietic stem cell
sUCBT	single umbilical cord transplant
T	Thymine
TAP	Transporter associated with antigen processing
TBE	Tris-Boric acid EDTA

TBI	Total Body Irradiation
TCR	T cell receptor
TCRA	T cell receptor alpha
TCRJ	T cell receptor joining
TCRV	T cell receptor variable
T_h	T helper cell
Thio	Thioteopa
TLR	Toll-like receptor
TNC	Total Nucleated cell
TNF-α	Tumour Necrosis Factor Alpha
Treg	Regulatory T cell
TREC	T cell receptor excision circles
UCB	umbilical cord blood
WBMT	Worldwide Network for Blood and Marrow Transplantation
µl	microliter

Chapter 1 : Introduction

1.1 The immune system

The human immune system is a complex system that consists of different molecules, cells and tissue types that interact with one another to protect the human body against damaging effects of pathogenic infection, toxins and cellular abnormalities. The immune system can be divided into two types: immunity and adaptive immunity (Abbas, 2017).

1.1.1 Innate immunity

Innate immunity is known as the natural immune defence system. This is a non-specific system that is the initial defence mechanism against invaders. Furthermore, physical and chemical barriers (complement, cytokines, epithelium and antimicrobial molecules synthesised by the surface membrane) work synergistically within innate immunity.

The human body has many first line barriers to prevent entry of pathogens. This includes epithelial surfaces such as the skin and the mucosa (mouth, gut and lungs). Within these barriers, proteins with antimicrobial properties and phagocytic cells are able to recognise microbes that penetrate epithelial barriers, which provide a secondary defence mechanism. In certain epithelial cell linings such as reproductive organs, respiratory and digestive tracts, there is secretion of mucus, which contains many factors such as enzymes, glycoproteins and peptidoglycans. These factors can protect epithelial cells from potential infections. (Russell and Mestecky, 2010, Turner, 2009, Knight and Holgate, 2003). Epithelial cells found within the respiratory tract and the digestive system express antimicrobial peptides such as cathelcidins and defensins. These protect the host by reducing and restricting the entry of pathogens (Skillman and Silen, 1972).

However, if pathogens can pass these physical barriers, they will interact with several phagocytic cells such as: dendritic cells (DCs), neutrophils, and macrophages. Phagocytes are the first cell type that can identify, engulf and destroy pathogens via a process called phagocytosis. This is the ingestion and digestion of microorganisms or foreign particles. Interactions between phagocytes and pathogens occur through receptors on the surface of phagocytic cells known as pattern recognition receptors (PRR), toll like receptors (TLR) and pathogen associated molecular patterns (PAMPS) found on the surface of pathogens (Dostert et al., 2008).

Phagocytosis of pathogenic cells can be induced via soluble plasma proteins within the blood known as complement molecules. These molecules can react with one another to make a foreign cell more susceptible to phagocytosis. Complement molecules can bind together to form a transmembrane pore on the pathogen known as the membrane attack complex. These transmembrane channels cause the pathogenic cell to lyse. A phagocytic cell will then identify and phagocytose the pathogen coated with complement molecules (Tomlinson, 1993).

Macrophages are cells that reside within the parenchyma; they are also one of the first cell types to be recruited to the site of infection and are long-lived. Additionally, neutrophils are high in population number at the site of infection, however they are short lived (Cavaillon, 1994, Cavaillon, 2001). Macrophages and DCs can also be known as antigen presenting cells (APCs) as they can express digested pathogen-processed antigens to T cells. In turn, this leads to the induction of an adaptive immune responses (Abbas, 2017).

Natural Killer (NK) cells play an important role in immunosurveillance. NK cells were originally described as granular lymphocytes that have natural cytotoxicity against tumour cells. Later, they were described as a separate lymphocyte lineage that have cytotoxic ability and can produce cytokines (Trinchieri, 1989). NK cells recognise transformed cells or infected cells and can kill these cells via antibody dependent cell cytotoxicity (ADCC). Furthermore, they can induce killing of target cells via the release of cytolytic granules such as perforin and

granzyme. NK cells release cytokines such as interferon-gamma (IFN-γ), which mediate critical roles in anti-tumour and anti-microbial responses. The responses initiated by IFN-γ includes: (1) initiating the transition of innate immune responses to adaptive immune responses via priming T cells; (2) stimulating the class switching of antibodies produced by B cells; (3) up-regulating the expression of adhesion molecules on epithelial surfaces and (4) promoting the interaction between leucocytes and epithelial cells (Schroder et al., 2004). IFN-γ has also been demonstrated to aid in ADCC (Delves and Roitt, 2000).

Once NK cells are stimulated, they also produce tumour necrosis factor alpha (TNF-α). This further enhances NK cell activity and cytotoxicity by upregulating the expression of adhesion molecules such as ICAM-1; in turn, this induces target cell lysis (Wang et al., 2012a).

1.1.2 The Adaptive Immune System

The adaptive immune system differs from the innate immune system and the main features of adaptive immunity are specific immunological reactions and the formation of immunological memory. It is a very specific system that can recognise different pathogens via an array of specific antigen receptors. (Bonilla and Oettgen, 2010). There are two essential types of adaptive immunity, known as humoral and cell-mediated immunity.

1.1.3 Humoral Immunity

Humoral immunity is a facet of immunity that is mediated by antibodies released by B cells. During humoral immunity, there is formation of immunological memory, which is the proliferation of B cells that produce antigen specific antibodies with a high specificity to foreign antigens. Antibodies are glycoproteins that are made up of two identical polypeptide chains called heavy chains and two shorter polypeptide chains known as the light chains. There are different classes of antibodies and they are categorised according to their heavy

chain type. This includes: immunoglobulin (Ig) A, IgD, IgE, IgG and IgM. Each type of antibody has a different function and is distributed differently throughout the body. Antibodies are required in humoral immunity to protect the host against extracellular microbes via recognition of microbe antigens, neutralisation of microbes and elimination (Abbas, 2017). Antibody production can take place in two ways; they are either secreted or bound to the membrane of plasma B cells as shown in Figure 1.1. However, both types of antibody are able to mount an effector function that involves the neutralisation of microbes, complement activation and induction of apoptotic pathways via ADCC (Murphy and Weaver, 2017). The humoral response is also characterised by long-lived antibody secreting cells with specific memory production that can respond to successive antigen exposure (Delves and Roitt, 2000).

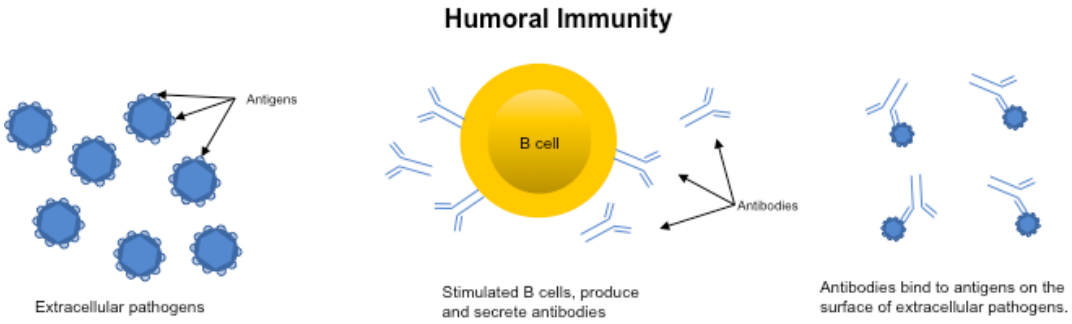


Figure 1.1 Humoral Immunity. Naïve B cells mature in to plasma B cells after recognising extracellular pathogens. This leads to the activation of plasma B cells and the secretion of antibodies that can bind to pathogens on the surface of extracellular pathogens. Subsequently, this leads to the elimination of the pathogen.

Naïve B cells circulating within the peripheral blood (PB) system express membrane bound antibodies, IgG and IgM, and mediate immune surveillance. Specifically, peptide immune responses take place when antigenic peptides are recognised by B cells residing within follicular lymphoid organs, which then go on to interact with T cells. This demonstrates that B cells have an APC function via human leukocyte antigen (HLA) class II molecules, to present antigens to T cells. However, for antigens to be presented to T cells, B cells must first internalise an antigen through a sequence of steps known as B cell activation. This activation is triggered through the binding of an antigen to the B cell

receptor (BCR). In turn, this initiates a cascade of intracellular signals that leads to the internalisation of the antigen, which is then processed and presented to T cells (Lanzavecchia, 1985).

B cells express two types of BCR known as IgM and IgD. The BCR consists of two regions: (i) membrane bound immunoglobulin (IgD, IgM, IgA, IgG or IgE) and (ii) an internalised signal transduction region Ig-α/Ig-β (CD79). CD79 has a cytoplasmic tail that is joined to an immunoreceptor tyrosine-based activation motif (ITAM) (Seda and Mraz, 2015). Upon activation of the ITAM motif, intracellular signalling cascade leads to the activation of various transcription factors, which translocate to the nucleus and induce class switching of antibody genes for antibody production. Class switching of genes involves an intricate process of gene reorganisation known as VDJ recombination, which takes place in somatic B cells. This occurs within the bone marrow (BM) and the variable; diversity and joining segments of the BCR are rearranged to give the BCR antigen specificity. The resulting effect is activation and proliferation of antigen-specific B cells that have the capacity to differentiate into antibody secreting plasma cells. Furthermore, different types of cytokines present during B cell differentiation will direct the class switching of heavy chains during antibody production (Mosmann and Sad, 1996).

1.1.4 Cell-Mediated Immunity

T cells play a fundamental role in cell-mediated immunity. They provide protection against intracellular pathogens including viruses and bacteria, which reside and proliferate within cells. T cells can be sub-classified into two types of cells: T helper cells (T_H or CD4+) or cytotoxic T cells (CTL or CD8+). For both types of T cells to mount a response, they require the recognition of cell-associated antigens. This leads to the proliferation of antigen-specific T cell clones which then differentiate into effector or memory T cells (Abbas, 2017).

T cell activation occurs through an interaction between highly specific T cell receptors (TCRs) and antigen presentation receptors known as HLA on APCs. TCR recombination takes place within the thymus during thymic selection. The TCR region is made up of an alpha and beta chain. For a functional TCR alpha-beta unit to be produced, multiple gene segments along the genomic DNA are joined and transcribed. Firstly, beta chains are rearranged and this is followed by gene rearrangement of the alpha chain (Chou et al., 1987a, Chou et al., 1987b). The TCR chain contains four regions encoded by the TCR-beta locus: variable, diversity, joining and constant region. Enzymes that mediate the recombination of the respective genes are known as RAG-1 and RAG-2 (Yancopoulos et al., 1986). RAG-1 and RAG-2 genes regulate the recombination of genes within the TCR-beta locus and recognise the specific recombination signal sequences (RSS), which are located before and after the diversity and joining sequences. Once this takes place, different variations of the variable region will join the diversity-joining region (Dudley et al., 2005). This produces a fully functional transcript of the TCR beta region and this is followed by the development and expression of a pre-TCR alpha complex, which is expressed on the surface of the thymocyte. This maintains the survival of the T cell and promotes the proliferation and progression to the double positive stage of T cell development within the thymus (Yamasaki et al., 2006). Upon T cell selection within the thymus, T cells are released into the periphery as naïve T cells.

HLA molecules are highly polymorphic and are composed of two classes: class I and class II. The expression of class I HLA molecules are on all nucleated cells and class II is expressed on macrophages, DCs and B cells. Both classes of HLA have a peptide-binding cleft on their outer surface, enabling presentation of antigens to T cells, Figure 1.2. CD4+ T cells are able to recognise antigens presented by HLA class II molecules while CD8+ T cells recognise antigens presented by HLA class I (Parkin and Cohen, 2001).

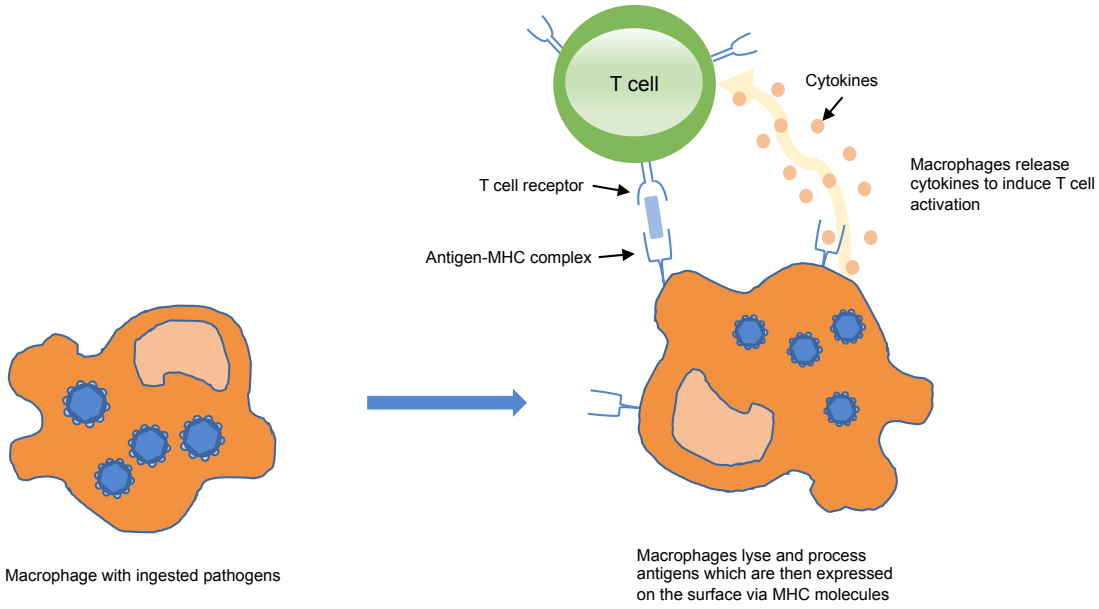


Figure 1.2 Cell mediated immunity. Cell mediated immunity where protection is formed against pathogens that cause intracellular infection. Infected cells present processed antigens to T cells, which are then primed and activated to proliferate and mount an immune response against infected cells.

Upon activation, CD4+ T cells differentiate and proliferate into different T cell subtypes such as T_h1, T_h2, T_h17 and inducible regulatory T cells (iTregs). T_h1 cells are vital in responses to intracellular microbes and secrete IFN-γ while T_h2 cells are important for responses to extracellular microbes and secrete interleukin (IL)-4 (Mosmann and Sad, 1996). T_h17 cells are protective against extracellular bacteria and secrete IL-17a, which has a fundamental role in autoimmune diseases and inflammation (Ouyang et al., 2008). Tregs are known to be immunosuppressive and regulate tolerance as well as immune homeostasis (Sakaguchi, 2005, Yamaguchi and Sakaguchi, 2006). T_h cells are also able to induce destruction of microbes and can mediate the recruitment of

phagocytic cells to the site of infection to stimulate macrophages and B cells through cytokine secretion (Bettelli et al., 2007, Stout and Bottomly, 1989).

In contrast to CD4+ T_h cells, CD8+ T cells and CTL cells can directly recognise pathogen antigens on infected cells or antigens on tumour cells presented by HLA class I molecules. Once CD8+ T cells are activated, they will acquire cytotoxic abilities and can directly facilitate killing of infected cells and tumour cells, as shown in Figure 1.3 (Abbas, 2017).

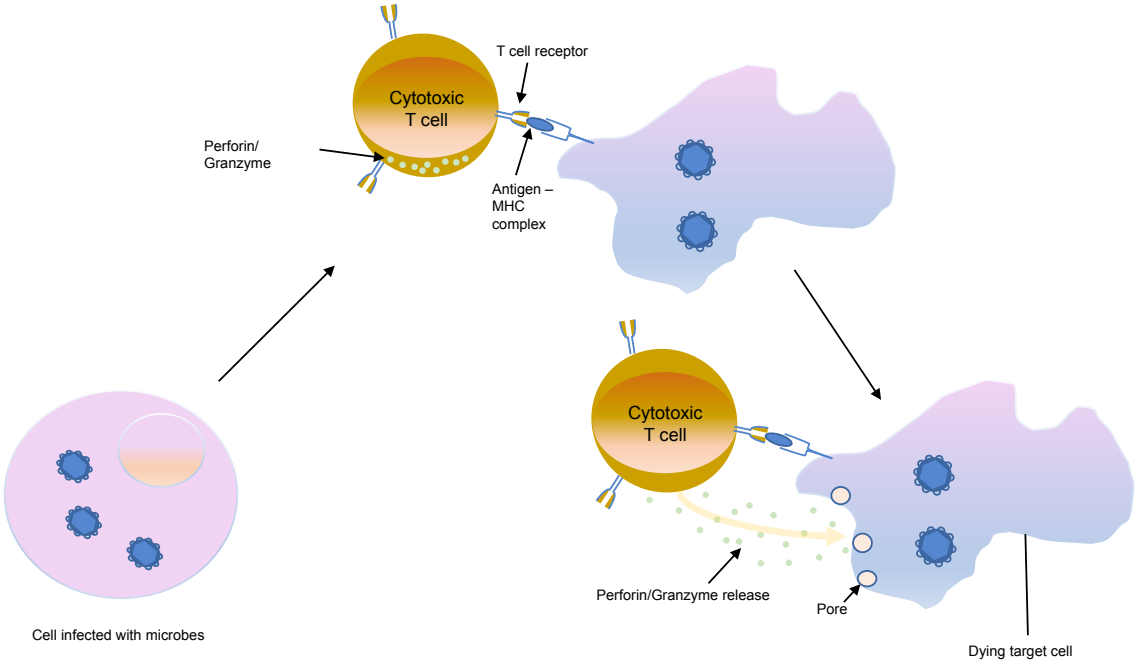


Figure 1.3 Cell mediated immunity. Protection is formed against pathogens that cause intracellular infection and there is direct lysis of the infected cell via cytotoxic T cells.

Once the pathogen has been eliminated, antigen specific T cells then undergo apoptosis and this restores immune homeostasis. A small proportion of T cells will differentiate and proliferate into long-lived memory cells able to rapidly respond to exposure of the same antigen. There are two types of long-lived memory cells: central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). T_{CM} produce high levels of IL-2 and express CD45RO, L-selectin and CCR7. This phenotype enables them to remain within lymphoid organs and induce clonal expansion of T cells after secondary exposure of pathogenic antigen. In contrast, T_{EM} express chemokine receptors such as CCR4, CCR5 and CXCR3.

These receptors enable T_{EM} to migrate into inflamed tissues (Sallusto et al., 2004).

1.2 Human Leucocyte Antigens

HLA are membrane bound glycoproteins that present processed antigenic peptides to T cells. Highly polymorphic genes located on chromosome 6p21.3 encode for the HLA system. There are two types of polymorphic HLA genes: class I and class II. Class I HLA molecules are expressed on all nucleated cells. However, class II HLA molecules are expressed on immune cells such as B cells, DCs and macrophages.

HLA class I molecules consist of two non-covalently linked polypeptide chains: 44-47 kDa heavy chain (HLA-A, HLA-B and HLA-C) and a 12 kDa beta 2-microglobulin, a non-HLA encoded subunit (Bjorkman et al., 1987). Degradation of intracellular proteins and foreign proteins takes place within the cytosol of cells. These are degraded by proteasomes into peptides. The peptides translocate to the endoplasmic reticulum (ER) via a transporter associated with antigen processing (TAP). Glycoproteins and chaperones then help form heterodimers within the ER before translocation of the HLA molecule to the cell surface (Vyas et al., 2008).

Class II HLA molecules are coded for by three polymorphic genes known as HLA-DR, HLA-DQ and HLA-DP (Stern and Wiley, 1994). The expression of class II molecules occurs on APCs, DCs and B cells and class II molecules bind to different antigenic peptides expressed by the respective cells. Formation of the class II molecule takes place within the ER, subsequently being transported to the endosomal compartment. The HLA class II molecule is then digested, leaving a residual associated peptide known as a class II-associated invariant chain peptide (CLIP). The class II peptides then bind to the groove of the HLA class II molecule and releases CLIP, which is regulated by HLA-DM and HLA-DO. The HLA class II molecule is then transported to the cell membrane where the antigen is expressed to CD4+ T cells (Kobayashi and van den Elsen, 2012).

1.3 Human Leucocyte Antigen Matching for Allogeneic Cord Blood Transplant

Histocompatibility testing is used to find a suitable HLA-matched donor for the recipient with the intention of reducing post-transplant complications that could arise due to HLA-incompatibility (Hirayama and Azuma, 2011). In the UK, 70% of patients of white Northern European descent can be fully HLA-matched with a donor. However, in the UK only 20% of non-white Northern European descent can be fully matched with a donor (Marsh et al., 2013, Hough et al., 2016).

In cord blood transplant (CBT), three HLA loci are matched between the donor and the recipient. This includes HLA-A, -B and –DRB1 (i.e. 6/6). HLA-A and HLA-B are matched at the antigenic level and HLA-DRB1 is matched at the allelic level. In CBT a patient should receive a 4/6 or better match for HLA-A, B, and DRB1 (Ballen and Lazarus, 2016). In comparison to BM or mobilized peripheral blood (mPB) transplant, HLA matching in CBT is less stringent as these treatments require high resolution tissue typing for HLA-A, -B, -C and -DRB1 (i.e. 8/8) (Barker et al., 2010).

1.4 Haematopoietic stem cell transplantation

1.4.1 Haematopoietic stem cells

Haematopoietic stem cells (HSC) are multi-potent cells that have the capacity to produce and maintain all types of blood cells and immune cells within the human body. Furthermore, HSCs maintain the respective blood cells throughout an individual's lifetime. They inhabit the BM and within this region they are able to continuously renew the populations of immune cells and blood cells.

As HSCs are self-renewing, they can be divided into two types of cells. These are known as short term HSCs (ST-HSC) and long term HSCs (LT-HSC) (Morrison and Weissman, 1994, Weissman, 2000). ST-HSCs have limited capacity to self-renew and have temporary haematopoiesis. However, LT-HSCs can maintain long-term self-renewal and haematopoietic restoration (Ho, 2005).

Both LT-HSCs and ST-HSCs can be defined by differential expression of cell surface markers. The process of haematopoiesis is sequential and LT-HSC will proliferate and differentiate into ST-HSC, which then differentiate into common lymphoid and myeloid progenitor cells. Common lymphoid progenitors can be identified via CD45 and CD7 cell surface markers. However, common myeloid progenitor cells are defined via the expression of CD45 and CD33 (Taussig et al., 2005). Subsequently, common lymphoid progenitors can differentiate into T cells ($CD3^+CD4^+$ or $CD3^+CD8^+$), B cells ($CD19^+$), and NK cells ($CD56^+$). Common myeloid cells will take a distinctive path of differentiation forming megakaryocyte/erythroid progenitors and granulocytes/macrophages known as MEP and GMP respectively. Furthermore, monocytes and neutrophils (myeloid cells) are generated from GMP. Red blood cells and megakaryocytes are produced from MEP (Weissman, 2015). HSC differentiation into different lineages is represented in Figure 1.4.

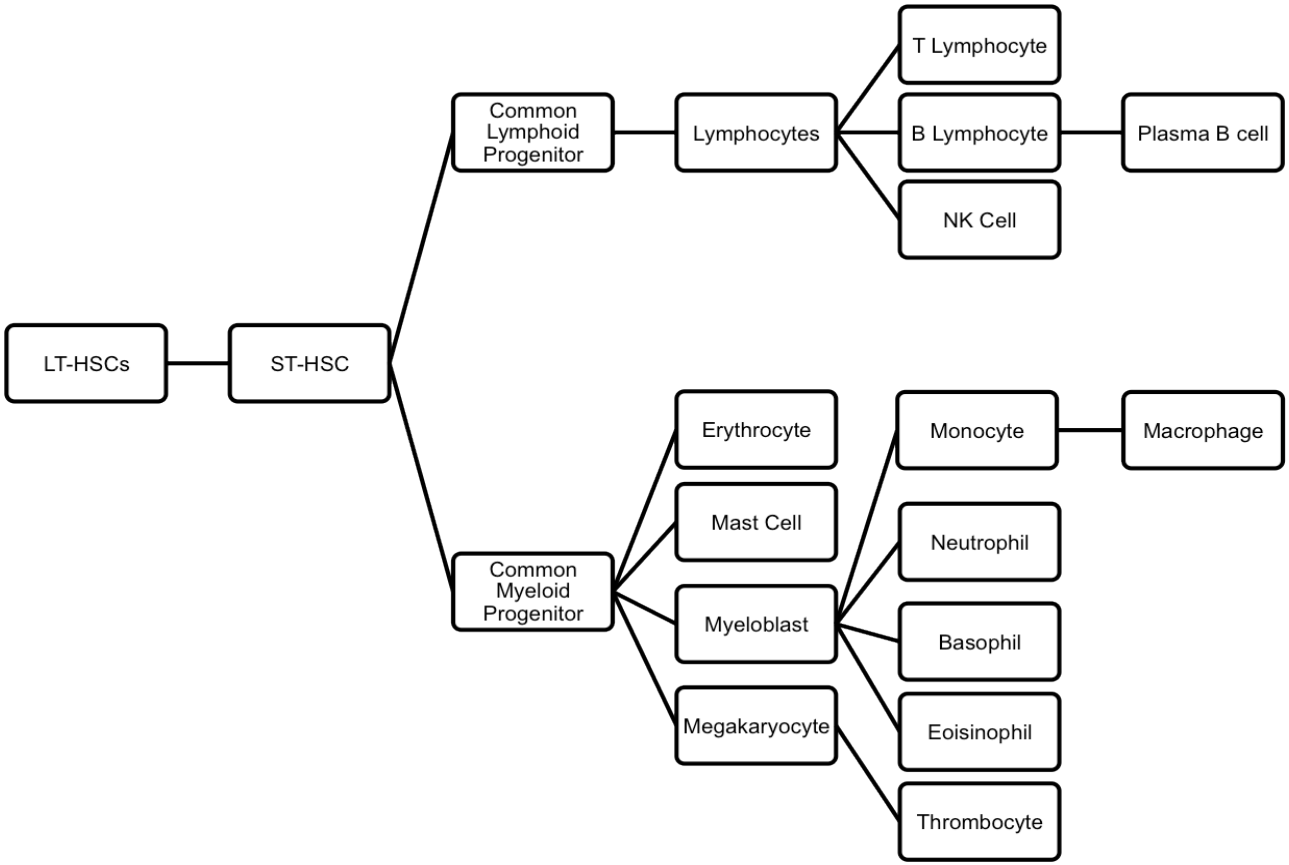


Figure 1.4 Haematopoietic stem cell differentiation, forming specialised blood cells. All blood lineages can be formed from long-term haematopoietic stem cells (LT-HSCs) that can generate short-term haematopoietic stem cells (ST-HSCs) that form common lymphoid and common myeloid progenitor cells. Subsequently these cells can produce all types of blood cells.

1.4.2 History of haematopoietic stem cell transplantation

The use of HSCs in haematopoietic stem cell transplantation (HSCT) originally comes from techniques used to repopulate cells within the BM. This was performed to reverse myelosuppression induced by radiation, which was seen in survivors of nuclear bombs in Nagasaki and Hiroshima, Japan. Nobel Laureate E. Donnall Thomas pioneered the technique of HSCT and performed the first syngeneic HSCT in 1956 (Thomas et al., 1975b, Thomas et al., 1957, Thomas et al., 1959, Thomas, 1975, Thomas et al., 1975a). Thomas and his respective colleagues pioneered HSCT by infusing BM from an identical twin to a recipient to treat a patient suffering from leukaemia in 1959 (Thomas et al., 1959). HLA was discovered in 1960 and the use of allogeneic HSCs became more practical. It was later discovered that HLA molecules are inherited, as haplotypes from both parents and the probability of two siblings to have HLA identity is one in four. This therefore led to Thomas and colleagues in 1977 to treat patients who suffered from end-stage leukaemia with BM of HLA-identical siblings (Thomas et al., 1977).

HSCT is mainly used to treat haematological diseases and cancers. More recently, HSCs have also been used to treat autoimmune diseases, immunodeficiencies and anaemias. Before HSCT can be performed, HSC must be acquired from a suitable source. A suitable source can be found who is a HLA matched donor, this is known as allogeneic. Allogeneic HSC can treat haematological diseases. However, there is a risk of developing complications post-transplant (Bensinger and Storb, 2001, Tabbara et al., 2002).

1.4.3 Haematopoietic stem cell sources

There are different sources of HSC that can be used for HSCT. BM has been the original source of HSC and is collected from donors under general anaesthetic from their iliac crests (Bahceci et al., 2000). Alternatively, mobilised peripheral blood (mPB) can be used whereby HSCs are harvested via apheresis after the donor has been treated with granulocyte colony stimulating factor (GCSF) (Copelan, 2006, Bensinger and Storb, 2001, Bensinger et al., 2001). More recently, cord blood (CB) has been used as an alternative source of HSC for transplantation. The use of CB originates from its initial use in 1988, where a patient with Fanconi's anaemia was infused with CB from a HLA matched sibling (Gluckman et al., 1989).

Haploidentical transplants are also considered to be a source of HSC and have been used in recent years. This is where the donor shares one HLA haplotype with the recipient and the patient is mismatched for HLA genes on the unshared haplotype. Donors for HLA-haploidentical transplant can either be biological parents, biological children, full or half siblings (Fabricius and Ramanathan, 2016). The pros of haploidentical grafts are that they can immediately be available and a graft can be mobilised within 10 - 12 days, which reduces the time to transplant compared to CBT, which can take several weeks. Haploidentical grafts have adequate doses of HSCs for transplantation and this includes adequate numbers of memory T cells, which are limited in CB grafts. Furthermore, there is immediate availability of the donor for repeated donations of HSCs in order to treat relapse within the patient, which is unavailable in CBT (Raiola et al., 2014). Respectively, the cost of graft acquisition in haploidentical transplant is lower compared to CBT. Haploidentical transplant also has major disadvantages and due to the high frequency of host and donor T cells being reactive to HLA alloantigens there is strong alloreactivity. Furthermore, this increases the risk of fatal graft rejection (Szydlo et al., 1997, Kindwall-Keller and Ballen, 2017). Additionally, in haploidentical transplant due to HLA disparity there is a high risk of GvHD and graft failure. However, when T cells are depleted from haploidentical grafts and post-transplant cyclophosphide is used

there is reduced incidence of GvHD but this could increase the risk of graft rejection and relapse (Ash et al., 1991, Barrett et al., 2011).

In 2016, a total of 1279 transplants were performed in the UK. This includes 33 BM transplants, 1210 mPB transplants and 36 CB transplants. (Source of information: http://bsbmt.org/activity/2016/). Furthermore, in 2016 a total of 3699 BM transplants, 13,481 mPB transplants and 437 CB transplants were performed (Source of information: https://www.ebmt.org/registry/transplant-activity-survey)

1.5 Early and delayed complications of haematopoietic stem cell transplantation

HSCT patients can be affected by complications post-transplant. The key issues faced post-transplant are: infections, graft versus host disease (GvHD), relapse, graft failure and delayed immune recovery. These are discussed below.

1.5.1 Delayed immune reconstitution and transplant related infections

Delayed immune reconstitution is one of the major issues faced after HSCT and is one of the major causes of infection in HSCT patients. Furthermore, the delay in immune reconstitution also leads to the increased risk of relapse of disease (Chakrabarti et al., 2002a, Chakrabarti et al., 2002b). The use of different conditioning agents, regimens and different sources of graft are contributors to the rate of immune reconstitution.

A number of studies have investigated immune reconstitution in HSCT. It has been shown that irrespective of the graft source, NK cells are the first lymphocytic cell type to reconstitute. The median day of neutrophil recovery is 14 days post-transplant (Komanduri et al., 2007, Beziat et al., 2009, Wu and Lanier, 2003, Szabolcs and Cairo, 2010). The reconstitution of NK cells, T cells and B cells reach normal healthy levels within the first three months post-transplant after BM transplantation (BMT) (Morecki et al., 2001, Fujimaki et al., 2001, Bartelink et al., 2013).

In contrast, the reconstitution of NK cells and CD8+ T cells in mPB recipients takes place within the first 100 days post-transplant. However, B cell and CD4+ T cell numbers have been shown to recover later and do so between 18 months to two years to reach the healthy adult range in mPB recipients (Fry and Mackall, 2005, Jacobson et al., 2012, Storek et al., 2001a).

Moreover, the reconstitution patterns of immune cells differ in CBT patients and are delayed in CBT patients compared to other graft sources. The first immune

cell type to reconstitute within CBT patients are NK cells, where patients reach normal healthy levels within three months post-transplant. Sequentially, B cells are the second type of immune cell to reconstitute within CBT patients, taking place between three and six months post-transplant (Komanduri et al., 2007, Beaudette-Zlatanova et al., 2013, Nakatani et al., 2014, Kanda et al., 2012).

T cell reconstitution is also delayed in CBT patients and the reconstitution of central memory and effector memory T cells takes place before naïve T cells (Komanduri et al., 2007). Furthermore, CD3+, CD4+ and CD8+ T cell numbers remain lower compared to healthy adults for up to one to two years post-transplant (Kanda et al., 2012, Jacobson et al., 2012, Komanduri et al., 2007).

The reconstitution of immune status after HSCT is affected by the type of progenitor cells infused into the patient and the types of conditioning regimen used prior to transplant are: myeloablative conditioning (MAC), reduced intensity conditioning (RIC) or non-myeloablative conditioning. Furthermore, the degree of histocompatibility between the recipient and donor (sibling, unrelated or mismatch) and types of GvHD prophylaxis (calcenurin inhibitors, mono/polyclonal antibodies or T cell depletion) can delay the reconstitution of immune cells post-transplant. In turn, this can lead to the development of opportunistic infection (Tomblyn et al., 2009).

Infection is a major issue that can arise after HSCT and three phases can be distinguished where specific pathogens infect HSCT patients at the respective phases. The three phases are known as phase I - pre-engraftment, phase II - post-engraftment and phase III - late phase, as shown in Figure 1.5. Conditioning can induce an initial period of pancytopenia immediately after HSCT (phase I). This is the phase where patients experience mucosal injury and neutropenia (Bearman et al., 1989). Patients within the first 30 days are at risk of bacterial infection from both gram-positive (e.g. Streptococcus, Staphylococcus and Staphylococcus aureus) and gram-negative (e.g. Enterobacter and Escherichia coli) bacteria. Additionally, during the first 100 days post-transplant, patients are also at risk of fungal infections such as Aspergillus, Candida and viral infections such as Herpes Simplex Virus.

(Balletto and Mikulska, 2015, Wingard et al., 2010). The damage caused to mucosal membranes and neutropenia is the major cause for these infections to arise within the pre-engraftment phase. Phase II is known as the post-engraftment phase. During this phase, HSCT patients will have immunodeficiency and reduced T cell and B cell functionality, which could be due to acute GvHD (aGvHD) development and aGvHD prophylaxis. In turn, this increases the chances of viral and fungal infections to develop. During phase II, the most common fungal infection is Candida albicans and the most common viral infections are cytomegalovirus (CMV), Epstein Barr Virus (EBV) and Adenovirus (Chakrabarti, 2007, Bitan, 2006, Tomblyn et al., 2009, Girmenia et al., 2011). Phase III takes place between 100 and 365 days post-transplant and many patients during this phase may be suffering from chronic GvHD (cGvHD) for which they are being treated. Respectively, during this time, patients are prone to fungal infections such as Candida and Aspergillus species (Marr, 2008, Girmenia et al., 2011). An overview of the various common infections in HSCT patients is detailed in Figure 1.5.

	Day 0 30	100	365	Reference
	Phase 1 Pre-engraftment	**Phase 2 Post-Engraftment**	**Phase 3 Late phase**	
Risks	- Neutropenia - Mucosal Injury - Reduced functional Immunity	- Decreased T cell and B cell numbers - Decreased T cell and B cell functionality - aGvHD - aGvHD treatment	- Decreased T cell and B cell functionality - cGvHD - cGVHD treatment	(Bearman et al., 1989)
Viral	Herpes Simplex Virus			(Tomblyn et al., 2009, Wingard et al., 2010)
		Adenovirus		(Chakrabarti, 2007)
		CMV		(Bitan, 2006, Farnault et al., 2015)
			EBV	
Fungal	Candida species			(Girmenia et al., 2011)
		Aspergillus Species		(Marr, 2008)
Bacterial	Gram Negative Bacteria			(Balletto and Mikulska, 2015, Ninin et al., 2001)
	Gram Positive Bacteria			

Figure 1.5 Types of common opportunistic infection in HSCT patients post-transplant.

1.6 Graft versus host disease

GvHD is a major cause of morbidity in HSCT patients (Ferrara et al., 2009). Bellingham *et al.* first distinguished GvHD in murine models of HSCT over 50 years ago (Billingham, 1966). The development of GvHD takes place when donor T cells respond to recipient tissue antigens presented by HLA molecules on the tissues of the recipient (Petersdorf et al., 1995). The risk of developing GvHD and its severity is associated with the level of HLA match/mismatch between the donor and the recipient (Kanda, 2013). Furthermore, mismatching of minor histocompatibility antigens can also lead to the development of GvHD (Goulmy et al., 1996). Antigen processing and post-transplant modification of peptides via HLA-molecules leads to donor T cells reacting to recipient antigens, which initiates GvHD (Goulmy et al., 1996).

1.6.1 Graft versus host disease classification

In 2005, the National institute of health (NIH) released new guidelines and a classification system to aid in the diagnosis of GvHD. Through these guidelines, two major classifications of GvHD are better known: aGvHD and cGvHD (Filipovich et al., 2005).

1.6.2 Acute graft versus host disease

Acute GvHD generally occurs in HSCT patients and is a reaction of the donor immune cells that react against the host cells. Commonly in aGvHD, the skin, liver and gastrointestinal tract (GIT) are affected and typically occur within the first 100 days post-transplant (Martin et al., 1990).

Ferrara and colleagues have detailed the pathophysiology of aGvHD as a three-stage process: phase 1, 2 and 3 (Hill and Ferrara, 2000). During phase 1, tissue damage and inflammatory response occurs causing the release of pro-inflammatory cytokines, such as IL-1, IL-6 and Tumour necrosis factor – alpha (TNF-α), from host tissues. This causes activation of neutrophils and

exacerbates pro-inflammatory cytokine release and chemokine production leading to increased inflammation (Hill et al., 1999, Ferrara et al., 2003, Ferrara et al., 2009). During this inflammatory response, MHC molecules become expressed on the host tissue and this leads to the activation of APCs. During phase 2, the induction and expansion phase, donor T cells become activated within the lymph nodes due to the presence of IL-12. T cells then secrete IL-2 and IFN-γ, which further recruits, expands and activates host-specific NK cells, CTLs and T_h (Jadus and Wepsic, 1992). Phase 3 is the final phase, where, effector functions take place through the migration of effector cells (NK cells and CTLs) and pro-inflammatory cytokines to the target organ. In turn, this induces localised target injury and destruction of the target tissue via interaction of CD95 to the CD95 ligand and the release of perforin and granzyme, which lyses the tissue (Nagata, 1994, Nagata and Golstein, 1995, Remberger et al., 2003).

1.6.3 Chronic graft versus host disease

Chronic GvHD is a multi-organ pathology that can develop in HSCT patients post-transplant. Chronic GvHD occurs in 60-80% of long-term survivors of HSCT (Nash et al., 2000, Ratanatharathorn et al., 1998). The common features of cGvHD are listed in the NIH guidelines (Jagasia et al., 2015). Many of the clinical features seen in cGvHD resemble clinical presentations within autoimmunity and immunodeficiencies such as scleroderma, lupus erythematous and Sjogren's syndrome (Snover, 1984, Shulman et al., 1980). The cause of cGVHD is complex and includes various factors such as: preceding development of aGvHD, age of the donor/recipient and the degree of HLA-mismatch between patient and donor (Carlens et al., 1998, Lee et al., 2002, Storb et al., 1983).

T cells have been shown to play a fundamental role in the development of cGvHD. Donor T cells play a central role in the attack against host tissue and the release of Th2 cytokines predominate in cGvHD (Sakoda et al., 2007). Additionally, donor B cells have been shown to have a potential involvement in the initiation and development of cGVHD. The homeostasis of B cells and

tolerance mechanisms are disrupted post-HSCT and this causes reduction in the formation of B cell memory. This leads to expansion of B cells that are activated by host antigens within the reconstituting B cell population (Allen et al., 2012, Corre et al., 2010, Greinix et al., 2008).

The severity of cGvHD is measured by clinical presentations that are graded using the NIH global grading system (Carlens et al., 1998). The clinical diagnosis of cGvHD is based on the differential presentations within HSCT patients that might occur. Differential sites of the body can be affected such as the oral cavity, skin, eyes, liver and GI tract. (Carlens et al., 1998, Aractingi and Chosidow, 1998, Serota et al., 1982, Shulman et al., 1988, Redding et al., 1998).

1.6.4 Prevention and treatment of graft versus host disease

As GvHD is one of the major complications post-transplant, prophylaxis is used to ensure that GvHD is controlled. Preventative measures are taken via the administration of immunosuppressive drugs.

In the prevention of GvHD, immunosuppressive agents are incorporated into reduced intensity and myeloablative conditioning protocols. The most commonly used agents for GvHD prophylaxis are listed in Table 1.1.

T cells play a vital role in the development of GvHD and immunosuppressive agents added to conditioning protocols are used to suppress T cell activity (Juliusson et al., 2006). Calcineurin inhibitors such as Tacrolimus and Ciclosporin A are frequently used to prevent the incidence of GvHD. Calcineurin inhibitors prevent the activation of nuclear factor of T cells (NFAT). Subsequently, this suppresses T cell expansion, which would be caused by IL-2 cytokine release (Solomon et al., 2003, Sabry et al., 2009). As part of preventative measures, antibodies are frequently used as part of conditioning regimes to prevent GvHD. The most commonly used are anti-thymocyte globulin (ATG) and CAMPATH-1H. ATG directly binds to T cells and induces T

cell depletion, whereas CAMPATH binds to CD52 present on mature lymphocytes inducing a wide spread depletion of lymphocytes within the HSCT recipient (Finke et al., 2009, Hale et al., 1998, Walker et al., 2016).

Agent	Mode of Action	Reference
Anti-thymocyte Globulin	Antibodies that recognise and bind to T cells	(Finke et al., 2009, Walker et al., 2016)
Ciclosporin A (CsA)	Calcineurin inhibitor and blockade of T cell action	(Solomon et al., 2003, Storb et al., 1986)
Cyclophosphamide	Cytotoxic agent that induces death of proliferating cells	(Colvin and Hilton, 1981)
Methotrexate	Interferes with metabolic activity of cells	(Storb et al., 1986)
Methylprednisolone	Lysis of lymphocytes via specific glycoprotein receptor binding	(Kanojia et al., 1984)
Mycophenolate Mofetil	Inhibition of DNA synthesis, inducer of lymphocyte apoptosis	(Mohty et al., 2004)
Monoclonal Antibodies (Alemtuzamab - CAMPATH)	Anti-CD52 binding on the surface of mature lymphocytes	(Hale et al., 1998)
Sirolimus	Blocks T cell and B cell activation	(Benito et al., 2001)
Tacrolimus	Calcineurin Inhibitor and blockade of T cell activation	(Sabry et al., 2009)

Table 1.1 Immunosuppressive agents used in the prevention of GvHD

Although HSCT recipients are administered pre-transplant GvHD prophylaxis, patients can still develop aGvHD and cGVHD. This has led to development of post-transplant treatment plans of GvHD using a number of immunosuppressive agents. The first line of treatment includes the use of steroids (Hockenbery et al., 2007, McDonald et al., 1998, Dignan et al., 2012a, Dignan et al., 2012b, Dignan et al., 2012c). However, long-term use of steroids can suppress the

immune system and lead to morbidity and mortality in HSCT patients. The most commonly used first line treatment plans are steroid combination therapies; methylprednisolone with or without calcineurin inhibitors (Martin et al., 2012).

There is no standard second line therapy for GvHD and treatment is guided on the basis of the grade and clinical presentation of GVHD. Second line options include the use of Imatinib (Glivec) and Sirolimus. Furthermore, the use of extracorporeal photopheresis (ECP) and new biological therapies are being used including Rituximab and Infliximab (Cutler et al., 2006, Olivieri et al., 2009, Sullivan et al., 1981). There are further possibilities for treatment of GvHD via the use of Thalidomide, Tacrolimus, Rapamycin and using pulsed doses of steroids. Additionally, patients could be administered total lymphoid irradiation in an attempt to reduce allo-reactive T cells.

1.7 Relapse

Relapse of the initial disease is one of the major causes of treatment failure and mortality in HSCT patients. Relapse can take place any time after transplant and can occur soon after HSCT. This could be due to the fact that resistant disease cannot be effectively eradicated by the conditioning regimen used prior to transplant. Relapse could also occur later due to a delay in immune reconstitution and weakness of the immune system (Komanduri et al., 2007, Murata et al., 2008).

1.8 Graft failure

Straight after HSCT, graft failure is a major complication that could take place. Recipient antibodies, T cells and NK cells are known to cause rejection of the graft through responses against donor haematopoietic cells. Additionally, other factors such as grafts with higher HLA disparities, unrelated grafts and T-replete grafts are also shown to be causes of rejection (Mattsson et al., 2008, Lamba et al., 2004). During graft failure, it has been demonstrated in murine models that T cells and NK cells become activated and proliferate within the recipient. This leads to responses against the donor's haematopoietic cells. Additionally, the source of graft is an important factor in graft failure development as it has been shown that BMT recipients have a lower chance of graft failure compared to CBT patients (Murphy et al., 1987, Raff et al., 1986, Kiessling et al., 1977, Cudkowicz and Bennett, 1971).

1.9 Graft versus leukaemia effect

Graft versus leukaemia (GvL) was first described in 1956 in a murine model. The initial concept was that donor marrow cells were responsible for the clearance of leukaemia (Barnes et al., 1956). A similar observation was seen in human BMT studies in 1965, where donor cells eliminated the leukaemia (Mathe et al., 1965). GvL is an important effect that has been shown to reduce relapse and could take place independently of GvHD (Gale and Horowitz, 1990). T cells have been shown to play a pivotal role in GvL effects. In particular, their responses to chronic myeloid leukaemia (CML) have been demonstrated after donor lymphocyte infusion (DLI) and allogeneic HSCT (Dickinson et al., 2017) Furthermore, T cells have also been demonstrated to have anti-tumour effects in HSCT patients against acute leukaemia or Myelodysplastic syndrome (MDS) (Daguindau et al., 2010). Respectively, NK cells have also been shown to play a critical role in GvL effects. A study conducted by Velardi *et al.* demonstrates donor versus recipient NK reactivity after acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) where patients were transplanted with a HLA-haploidentical mismatched family donor (Velardi, 2008).

1.10 Conditioning regimens used for haematopoietic stem cell transplantation

HSCT recipients are prepared with chemotherapy and/or radiotherapy. The principle of undertaking chemotherapy is to ablate the malignant cells within the patient therefore eradicating disease. Conditioning also serves various purposes such as prevention of graft rejection and development of GvHD. Conditioning therefore prepares the patient for engraftment of HSCs, which will later be infused into the patient. However, it is also known that conditioning can cause mortality due to toxicity related complications (Vriesendorp, 2003, Santos et al., 1983).

1.10.1 Types of conditioning regimens

There are three main groups of conditioning regimens that are used pre-transplant: myeloablative conditioning (MAC) (with or without total body irradiation (TBI)), reduced intensity conditioning (RIC) and non-myeloablative conditioning (NMA) (Bacigalupo et al., 2009).

1.10.2 Myeloablative conditioning

One of the earliest studies by Thomas *et al.* has demonstrated the administration of a BM graft to patients receiving radiation and chemotherapy (Thomas et al., 1957). Myeloablation was first tested in murine models, where mice were exposed to 10 gray (Gy) of TBI. These were shown to have pancytopenia, which was recovered by BMT (Lorenz et al., 1952). Myeloablation causes complete haematological destruction and a patient must be followed up with a HSCT to recover their haematopoiesis. Myeloablation is used to eradicate the haematological or non-haematological disease and to suppress the recipient's immune system, thus preparing the recipient for HSCT. The main chemotherapeutic agents used to prepare patients for HSCT using the MAC regimen are TBI, Cyclophosphamide (CY) and Busulfan (Bu) and the combination of CY-TBI or BU-CY common myeloablative conditioning regimens.

In MAC, other high dose chemotherapeutic agents such as Dimethylbusulfan, Etoposide, Thioteopa (Thio) and Melphalan (Mel) can also be used in combination with CY or TBI (Terenzi et al., 1996, Kanfer et al., 1987, Jamieson et al., 2003, Helenglass et al., 1988).

1.10.3 Reduced intensity conditioning

This conditioning protocol was set up to reduce toxicity in HSCT patients. Reduced intensity regimens have been formed as many studies supported the fact that lower doses of chemotherapy are associated with successful engraftment in related and unrelated donors (Giralt et al., 1997, Niederwieser et al., 2003). Furthermore, these regimens also demonstrated that older patients suffered reduced toxicity and a stable GvL effect was established (Niederwieser et al., 2003). RIC regimens cause cytopenia that can be prolonged in patients and this means that patients require HSCT. The TBI or alkylating agents are reduced in RIC regimens. RIC regimens can include Fludarabine (Flu) with other alkylating agents such as Mel, Bu, and Thio (Tauro et al., 2005, Corradini et al., 2005, Slavin et al., 1998).

1.10.4 Non myeloablative conditioning

It has been demonstrated that transplant related mortality increases if myeloablative conditioning is used and it also increases with the patient age (Jamieson et al., 2003). With this in mind, NMA conditioning regimens were devised and are predominantly used in older patients. NMA regimens cause little cytopenia and have a very low toxicity. However, they have an immunosuppressive capacity. When NMA regimens are combined with mPB transplant, the results have shown that patients have complete donor engraftment. Common combinations of NMA regimens are Fludarabine and Cyclophosphamide (Flu-Cy), Total lymphoid radiation (TLI), TBI at 1 Gray (Gy), TBI at 2 Gy and ATG (Ballen et al., 2004, Storb, 2001, Lowsky et al., 2005, Khouri et al., 1998). Patients administered with NMA regimens usually have delayed GvHD development and this normally takes place after 100 days post-

transplant. However, GvHD is a significant cause of morbidity and mortality in NMA conditioned patients (Mielcarek et al., 2003).

1.11 Cord blood transplantation

1.11.1 The history of cord blood transplantation

The use of cord blood (CB) was first proposed as an alternative source of HSCs for transplantation in 1982. Subsequently, Gluckman *et al*. performed the first related CBT in 1989 to a patient with Fanconi's anaemia (Gluckman et al., 1989). The patient had complete engraftment with donor cells and has remained in haematological remission for more than 20 years without any GvHD development. Following these promising results in related CBT, an unrelated CBT was first performed in 1993 in a patient suffering from refractory leukaemia (Kurtzberg et al., 1996). Furthermore, a paediatric study performed by Wagner *et al.* demonstrated that cryopreserved CB units could be used as a safe source of transferable HSC in clinical transplantation. Additionally, Wagner *et al.* demonstrated that CB contains adequate CD34+ cells, which can aid in replacing the haematopoietic system (Wagner et al., 1996). Since then, much work has been undertaken to understand the immunology of umbilical cord blood (UCB) and refinement in the procedures of collection, processing and preservation and thawing techniques.

1.11.2 Cord blood banking

The first cord blood bank (CBB) was established in 1993 in New York, Milan and Dusseldorf and this was followed by the first CBB bank in the UK in 1996 (Bradley and Cairo, 2005). There are various bodies that govern the quality and standards of CB units collected. Organisation such as NETCORD, Eurocord, FACT and the European Blood and Marrow Transplantation society ensure that standards are met for the volumes of CB collected, sterile processing of the CB unit, the measurement of CD34+ counts within the CB unit, cell recovery methods after processing and red blood cell depletion of the CB unit. It has been reported by the Bone Marrow Donors Worldwide (BMDW) that there are 36 countries worldwide that contain a total of 53 cord blood registries.

Furthermore, there are 721,289 CBU registered (as of August 2017) (https://www.bmdw.org).

1.11.3 Advantages and disadvantages of cord blood transplant

Over the last 25 years, CBT has become a source of HSC increasingly used for HSCT. CB is easily obtained from the donor and no harm is caused to the donor when collecting a CB sample. The CB sample is cryopreserved after collection and when required is easily available. Compared to mPB and BMT recipients, CBT patients require less stringent HLA matching between the donor and the recipient, this is due to the naivety of the CB cells. This therefore makes CB a viable source of HSC for patients who are unable to find a related or unrelated BM or PB donor (Gluckman et al., 1989, Broxmeyer et al., 1989). Furthermore, the processing and delivery of CB into the recipient can be undertaken easily in comparison to PB and BM harvests and there is a reduced risk of the transfer of infections from the donor to the recipient. CBT patients have also shown to have a lower incidence of GvHD even though there can be CBT with tolerable higher degree of donor-recipient HLA-mismatches (Barker et al., 2010, Rocha et al., 2004).

Despite the advantages, the use of CB faces drawbacks. It has been demonstrated that there is a low cell dose within the CB unit compared to mPB and BM grafts. Additionally, CBT patients cannot be given a DLI post-transplant. Furthermore, the use of CB is associated with an increased risk of graft failure and delayed immune reconstitution (Rocha et al., 2004, Laughlin et al., 2001). CBT patients have also been shown to have a delay in the time to neutrophil and platelet engraftment when compared to BMT and mPB transplant patients. The median time to neutrophil engraftment is 30 days in CBT patients, 21 days in BMT patients and 14 days in mPB transplanted patients (Seggewiss and Einsele, 2010). The delay in immune reconstitution could therefore lead to a higher risk of infection post-transplant, which leads to transplant related mortality (TRM) (Laughlin et al., 2001, Laughlin et al., 2004).

As a result of delayed immune reconstitution in CBT, the most common infection is CMV and EBV (Locatelli et al., 1999, Wagner et al., 2004).

1.11.4 Single versus double cord blood transplant

Single CB units were first used to treat paediatric patients diagnosed with haematological and non-haematological diseases (Locatelli et al., 1999, Locatelli et al., 2003). CB contains a higher concentration of HSCs. However, each CBU contains fewer total nucleated cells (TNC) by one or two logs compared to BM or mPB grafts (Rocha and Broxmeyer, 2010). Studies have shown that the use of single CBU has been demonstrated to account for long-term haematopoiesis in 80% of transplanted patients within 30 days of transplant and 100% of patients by 365 days post-transplant (Brunstein et al., 2007). Furthermore, a study comparing single CB treated patients versus BM transplanted patient's shows that the clinical outcomes (relapse, overall survival, leukaemia free survival, total relapse mortality and GvHD) of CBT patients are similar to that of BMT patients (Atsuta et al., 2009). The CD34+ cell dose and the TNC dose are important factors associated with the probability and rate of neutrophil engraftment. Due to the lower TNC count in a single cord blood unit (sCBU), the infusion of double cord blood units (dCBU) have been used to increase the number of TNC infused into patients, known as double umbilical cord blood transplant (dUCBT) (Cornetta et al., 2005, Gluckman et al., 1997, Laughlin et al., 2004, Wagner et al., 2002, Rubinstein et al., 1998). There is an overall consensus that a recipient should be infused with a minimal TNC dose of $2.5x10^7$/kg of the recipient's body weight and should be matched with at least 4/6 HLA matches per unit (Barker et al., 2001, Barker et al., 2005, Hough et al., 2016, Snowden et al., 2016). Since there is a higher TNC content by using dUCBT, clinical studies have also shown that there is improved engraftment with a reduced risk of relapse and severe aGvHD (Barker et al., 2003, Barker et al., 2005).

1.11.5 Cord blood unit dominance

The use of dUCBT has decreased the rate of graft failure and TRM in adults and this has led to further understanding of the biology post-transplant as it has been noted that one CBU is dominant over the other and serves as source of long-term haematopoiesis (Barker et al., 2005, Brunstein et al., 2007, Ballen et al., 2007). The biology of unit dominance remains unclear and factors such as HLA-disparity, sex mismatch, TNC count, CD34+ count and viability have not been reliable predictors of which unit will be dominant over the other (Majhail et al., 2006). However, it has been demonstrated CD34+ cells with viability less than 75% infused into recipients is associated with poor engraftment (Scaradavou et al., 2010).

1.11.6 Methods to improve outcomes of cord blood transplant

Many of the techniques used to improve the outcomes are used to induce engraftment and to improve immune reconstitution following CBT, as detailed in Table 1.2.

	Technique	Reference
Modification of conditioning regimens	- T-cell depletion protocols - Reduced intensity conditioning	(Eyrich et al., 2001) (Barker et al., 2003)
CB unit selection	- KIR matching - Detection of donor specific antibodies	(Willemze et al., 2009) (Takanashi et al., 2008)
CB cell dose	- Improved collection, processing and freezing and thawing - Infusion of two CBU - Infusion of CB with third party donor cells - *Ex vivo* expansion of CB cells	(Naing et al., 2015) (Rocha et al., 2010) (Bautista et al., 2009) (Mohamed et al., 2006)
Delivery and homing of HSCs	- CD26 inhibition - Intrabone infusion of CB - Fucosylation of HSC within CB units	(Cutler et al., 2013) (Frassoni et al., 2008) (Popat et al., 2015)
Post-transplant cytokines	- Administration of Granulocyte colony stimulating factor (GSCF) - Administration of stem cell factor (SCF)	(Gotoh et al., 2014) (Mohamed et al., 2006)
Use of accessory Cells	- The use of Mesenchymal stem cells - The use of Tregs	(Luan et al., 2015) (Brunstein et al., 2011)
Transplant protocol modifications	- Reduced intensity protocols	(Rocha et al., 2009)

Table 1.2 Improving outcomes of cord blood transplant

Various approaches have been used to improve the time to engraftment and immune reconstitution in CBT patients. However, further studies are required to better understand the kinetics and diversity of reconstituting immune cell subsets. This will provide a better insight into the reconstitution of immune cells within CBT patients, which will aid in guiding clinical decisions and post-transplant therapeutics. Additionally, this will provide alternative pathways that

could be researched to improve the time to engraftment and immune reconstitution. In this current study, the focus has been on immune reconstitution in CBT patients. A detailed description is discussed in the following sections.

1.12 Immune reconstitution in cord blood transplant

Monitoring immune reconstitution in CBT could allow the identification of the cell types involved in the recovery of the haematological system. By measuring the kinetics and diversity of recovering immune cell subsets, cell types can be identified which could influence the exacerbation of post-transplant complications such as relapse, GvHD and infections. Immune reconstitution is the time taken for immune cells to replenish and for the immune system to recover within a patient once they have undergone HSCT. Patients who undergo CBT are known to experience delayed engraftment and poor immune reconstitution. This has been compared to a number of BM and mPB studies as discussed previously.

1.12.1 Immune cells within the cord blood graft

1.12.1.1 Monocytes in cord blood grafts

Little is known about the absolute numbers of monocytes within CB grafts. However, it has been demonstrated that monocyte absolute counts within CB grafts are similar to absolute counts recorded in BM grafts. Furthermore, the number of monocytes and the frequency of monocytic populations are similar to that seen in adult PB (Sohlberg et al., 2011, Sorg et al., 2001).

1.12.1.2 NK cells in cord blood grafts

Within CB grafts there is a high proportion of NK progenitor cells such as $CD16^{positive}CD56^{negative}$. NK cells within the CB graft are mainly of two subtypes: $CD56^{bright}$ and $CD56^{dim}$. These NK cell populations are in similar proportions to NK cells within adult PB (Gaddy et al., 1995, Verneris and Miller, 2009). NK cells within CB grafts have a low level expression of adhesion molecules. This is supported by the fact that NK cells within CB grafts highly express NKG2A, an inhibitory receptor and a low level expression of granzyme B. This could

mean that NK cells engrafted into patients may have decreased cytotoxic activity (Tanaka et al., 2003, Wang et al., 2007).

1.12.1.3 B cells in cord blood grafts

CB grafts contain B cells that are predominantly naïve characterised by the expression of CD5 and IgM. Furthermore, they have low-level expression of CD23 compared to adult PB, which is a low affinity receptor for IgE. This receptor plays an important role in parasitic infections and allergic reactions (Szabolcs et al., 2003). It has been demonstrated that CB B cells have a high number of poly-reactive IgM antibodies. However, they have a decreased capacity to class switch, which leads to a decrease in the IgA and IgG antibodies produced by CB B cells (Durandy et al., 1990). In addition, B cells within CB grafts have an increased susceptibility to lyse via apoptosis (Kessel et al., 2006).

1.12.1.4 T cells in cord blood grafts

The majority of CD4+ and CD8+ T cells within the CB graft are considered to be naïve as compared to BM and PB grafts, which contains a higher proportion of memory T cells within the graft. Furthermore, the majority of CB T cells have a naïve phenotype and have not been challenged by antigens. Overall, this means that CB T cells are considered to be less alloreactive compared than adult donor T cells. CB contains a lower absolute numbers of CD4+ and CD8+ T cells compared to BM and mPB (Szabolcs et al., 2003). The proliferation of T cells after CBT has been shown to occur rapidly, which is particularly caused by allogeneic stimulation. However, CB T cells have limited antigen specific cytotoxicity post-transplant (Risdon et al., 1994). Despite the limited antigen-specific cytotoxicity, CB T cells attain an enhanced anti-tumour function against B cell lymphoma compared to PB T cells, which has been shown in murine models (Hiwarkar et al., 2015).

Moreover, there is a high proportion of Tregs (CD4+CD25+FoxP3+) within the CB grafts; these cells have a highly suppressive function (Godfrey et al., 2005). CB Tregs have a naïve phenotype and they predominantly express CD45RA and low expression of CD45RO in comparison to Tregs from healthy adult PB (Chang et al., 2005). Furthermore, CB Tregs have a higher rate of expansion after being stimulated with anti-CD3 and anti-CD28 antibodies. This highlights a potential of Treg expansion, where Tregs might be able to expand *in vivo* post-transplant (Torelli et al., 2012).

1.12.2 Platelet and neutrophil engraftment in cord blood transplantation

Following conditioning chemotherapy and/or TBI and the infusion of CB, there is an initial phase of aplasia. During this phase, CB HSCs engraft, proliferate and expand within the BM of the recipient. TNC counts of the graft are an important factor for efficient engraftment in CBT patients and it has been demonstrated that increased CD34+ cells and higher TNCs are associated with an increased probability and rate of neutrophil and platelet engraftment (Danby and Rocha, 2014, Gluckman et al., 2004).

CB contains lower TNC doses compared to BM and mPB grafts. The time to neutrophil engraftment is defined as the first three consecutive days with a neutrophil count greater than 0.5 x 10^9/L. Comparatively, it has been seen that the median time to neutrophil engraftment in CBT patients is 30 days, which is slower compared to BM and mPB recipients where a median time to engraftment is 21 days and 14 days, respectively (Seggewiss and Einsele, 2010). The time to platelet recovery is recorded as the first three consecutive days with a platelet count greater than 20 x 10^9/L. It has been observed that the time to platelet recovery is also delayed in CBT recipients compared to BM and mPB recipients and the time to platelet engraftment can range between 50 days to 100 days post-transplant (Nakasone et al., 2016, Storek et al., 2001a, Seggewiss and Einsele, 2010).

1.12.3 NK cell reconstitution in cord blood transplantation

NK cells are one of the first lymphocytic populations that reconstitute in CBT patients. In adult CB recipients, the time to NK cell reconstitution has been observed as 30 days post-transplant (Brahmi et al., 2001, Jacobson et al., 2012, Ruggeri et al., 2011). It has been demonstrated that CBT recipients have both populations of NK cells $CD56^{dim}$ and $CD56^{bright}$. However, there are discrepancies in the reconstitution of NK cell populations within CBT recipients. Early post-transplant, the reconstitution of NK cells in CBT patients occurs through the expansion of $CD56^{bright}$ NK cells and absolute numbers return to the healthy range within three months post-transplant (Somers et al., 2013, Ruggeri et al., 2011). Following the initial three months, there is an increase in the proportion of $CD56^{dim}$ NK cells and this coincides with an absolute count increase in the number of $CD56^{dim}$ NK cells (Cooley et al., 2005, Nguyen et al., 2008). There is a temporary increase in the absolute counts of NK cells, which exceeds the normal range of NK cells compared to healthy controls. This could occur to compensate the T cell lymphopenia post-CBT (Komanduri et al., 2007). Additionally, the reconstitution of NK cells has been demonstrated to occur through NK cells from the predominant unit in dUCBT patients and reconstitution of NK cell subsets can occur between 180 days to 365 days post-transplant (Ruggeri et al., 2011, Somers et al., 2013).

1.12.3.1 NK cell function in cord blood transplantation

$CD56^{dim}$ NK cells are known to have a higher cytotoxicity compared to $CD56^{bright}$ NK cells (Cooper et al., 2001, Nagler et al., 1989). In HSCT, NK cells could play a role in GvL. T cell reconstitution is delayed and this means that the clearance of infection and residual diseases could be delayed. However, NK cells could compensate for the delay in T cell reconstitution and are shown to be key mediators of GvL responses, which have been demonstrated in haplo-identical transplantation (Ruggeri et al., 2002). In CBT studies, it has been demonstrated that the reconstituting NK cells are fully functional and mature within the first three months post-transplant. NK cells in CBT patients are able

to lyse leukaemic blast cells through direct lysis and through ADCC pathways (Beziat et al., 2011, Beziat et al., 2009). However, few studies have investigated the function of NK cells post-transplant and further work is required to elucidate the functional activity of NK cells in CBT patients.

1.12.4 B cell reconstitution in cord blood transplantation

B cell reconstitution occurs between 100 and 180 days post-CBT. Comparatively, the reconstitution of B cells is faster compared to BM and mPB recipients (Beaudette-Zlatanova et al., 2013, Kanda et al., 2012, Komanduri et al., 2007). A study undertaken by Nakatani *et al.* demonstrates that reconstitution of CD19+ B cells takes place in the first three months post-transplant in adult recipients (Nakatani et al., 2014). However, through BMT and mPB transplant studies, there is an increase in the absolute number of B cells and within the first three months post-transplant (Beaudette-Zlatanova et al., 2013, Jacobson et al., 2012, Kanda et al., 2012, Mehta and Rezvani, 2016). Little is known about B cell subset reconstitution in CBT of adults. Additionally, HSCT patients have an increase in the absolute numbers of transitional B cells (CD19+CD24+CD38+). This leads to an increase in the absolute numbers of mature B cells (CD19+CD24+CD38-) in HSCT patients (Marie-Cardine et al., 2008). Overall, the increase in the absolute number of B cells, in CBT patients, also exceeds the range of B cells seen within healthy adults, which could be due to the T cell lymphopenia experienced by patients post-transplant (Komanduri et al., 2007).

During B cell development within the BM, B cells arise from undifferentiated haematopoietic precursors. B cell development is split into two phases: the initial antigen-independent phase, where precursor B cells mature into functional B lymphocytes and the antigen-dependent phase, where mature B cells are generated through selection processes (Ghia et al., 1998). During B cell maturation within the BM, kappa deleting recombination excision circles (KRECs) are formed. These are bi-products of allelic exclusion of the Ig kappa locus (IGK) (van Zelm et al., 2007, Siminovitch et al., 1985). KRECs are formed

when B cells rearrange the IGK and within B cells the Ig kappa locus becomes non-functional and is deleted from the immunoglobulin constant gene (IGC). The IGK is located 24 kilobases (kb) downstream from the IGC gene segment. During gene re-arrangement, the IGK is excised from the IGC and recombined within a RSS, which is located on either side of IGK variable gene segment. After the IGK recombines with the RSS, there is formation of a coding joint that prevents any further attachment of the IGK within the IGC locus. Thus, the IGK is excised from the DNA forming a KREC excision loop (van Zelm et al., 2007, van Zelm et al., 2011, Beishuizen et al., 1997). KRECs are found in high copy numbers in naïve/transitional B cells that are released into the periphery. The copy numbers of KRECs become diluted as B cells proliferate within the periphery.

The detection of KRECs can be utilised to understand the output of B cells from the BM, which has been demonstrated in a number of CBT studies and HSCT studies (Nakatani et al., 2014, Chiarini et al., 2013, Serana et al., 2013). The quantification of KRECs has shown that reconstitution of B cells occurs as early as one month post-CBT. Furthermore, at three months post-CBT, patients have the highest level of KRECs. This indicates that BM output of B cells occurs within the first three months post-transplant (Nakatani et al., 2014).

1.12.4.1 B cell subset reconstitution in cord blood transplantation

The number of B cells that reconstitute within CBT patients occurs within three to six months post-transplant and the absolute counts reach the healthy adult ranges within a median time of six months (Renard et al., 2011, Kanda et al., 2012, Servais et al., 2014). KREC assays have been utilised to demonstrate that B cell reconstitution is enhanced in CBT recipients compared to mPB and BMT (Nakatani et al., 2014). The reconstitution of B cells is quicker compared T cells and T cell subsets (Bemark et al., 2012). The functional reconstitution of B cells is dependent on the reconstitution of a number of B cell subsets within CBT patients. During the first three months post-transplant, there is an increased circulation of transitional B cells within the periphery (Marie-Cardine

et al., 2008). The early circulating B cells are BM emigrants that have a very low BCR specificity and a partial response to antigen stimulation (Storek et al., 1993). Gradually, over the subsequent months, transitional B cells and immature B cells differentiate into mature B cells. Jacobson *et al.* demonstrated that dCBT recipients have quicker recovery of IgG compared to mPB recipients and the levels of IgG recover between five and six months post-transplant. This therefore demonstrates that B cell functionality and the capacity to produce antigen specific antibodies occurs within the first year post-CBT (Jacobson et al., 2012). However, it has been reported that long-term functional maturation of B cells could take longer than one year as most of the circulating B cells are of a naïve phenotype (Charrier et al., 2013, Servais et al., 2014). Limited data exists on the crucial developmental stages for B cell maturation in CBT patients.

1.12.5 T cell reconstitution in cord blood transplantation

T cells reconstitution is delayed CBT patients, taking between six months and two years post-transplant. The reconstitution of T cells is dependent on different pathways known as the thymic independent and the thymic dependent pathways (Williams et al., 2007).

1.12.5.1 Thymic independent reconstitution of T cells

Early post-transplant adoptively transferred T cells proliferate within the recipient. Upon exposure to antigens, T cells proliferate within the recipient (Mackall et al., 1996). In dUCBT, it there are elevated levels of IL-7 in CBT patients for up to two years (Politikos et al., 2015). IL-7 plays an important role in regulating the thymic independent expansion of T cells and IL-7 levels are inversely correlated with absolute counts of CD4+ T cells (Politikos et al., 2015). T cells within the CB graft are predominantly naïve and have limited TCR diversity. This limits their ability to recognise a plethora of self and non-self antigens compared to healthy adult T cells. Therefore, CBT recipients are unable to provide sufficient protection against pathogens, early post-transplant, due to limited memory T cell formation (Chalandon et al., 2006). During homeostatic peripheral expansion (HPE), T cells within the graft change their characteristics from a naïve phenotype to an effector memory and central memory phenotype (Mackall et al., 1993). This is a phenotypic and functional change. In a study undertaken by Komanduri *et al.* there is skewing of the T cell phenotype, where there is a higher proportion of memory and central memory T cells in dUCBT recipients. Furthermore, Komanduri *et al.* investigated the kinetics and diversity of T cell reconstitution in CBT patients, looking at the specific reconstitution patterns of CD4+ and CD8+ naïve T cells (CD45RA+CCR7+), effector T cells (CD45RA-CCR7-) effector memory T cells (CD45RA+CCR7-), and central memory T cells (CD45RA-CCR7+). The findings from this study showed that there is delayed reconstitution of naïve T cells and an increase numbers of effector memory T cells post-transplant. Furthermore, T

cells have an impaired functionality in responses to CMV peptides (Komanduri et al., 2007).

As CBT recipients have delayed T cell reconstitution, it has been shown that T cells that proliferate via the thymic independent pathway are prone to apoptosis. This could reduce the cell number of T cells within the periphery post-transplant. As a result, this could lead to a reduced TCR repertoire and limit the antigenic specificity in CBT patients, which could be one of the main causes as to why there is a high prevalence of infection early post-transplant (Hakim et al., 1997, Roux et al., 1996).

Additionally, Tregs are considered an essential component of central and peripheral tolerance of the immune system (Sakaguchi et al., 2009). Tregs have suppressive and immunomodulatory effects and have a positive correlation with preventing the exacerbation of immune responses, thus preventing tissue injury (Di Ianni et al., 2011, Misra et al., 2004). In CBT recipients, the absolute numbers of Tregs are low for up to six months post-transplant (Jacobson et al., 2012).

1.12.5.2 Thymic dependent reconstitution of T cells

Long-term immune reconstitution of T cells is also dependent on the development of T cells through the thymus. Through this pathway, there is generation of naïve T cells with a broad TCR diversity and ability to recognise a variety of antigens (Politikos and Boussiotis, 2014, Roux et al., 2000). In order for thymic production of T cells to take place, lymphoid progenitors from the graft must engraft within the recipient and migrate to the thymus. In the thymus, they will interact with cortical thymic epithelial cells and undergo maturation to undertake lineage commitment. Notch signalling induces lineage commitment within T cells and induces their differentiation and commitment to naïve T cells (De Smedt et al., 2002).

During thymic development of T cells, there is rearrangement of the TCR, as described in section 1.1.4. Gene rearrangement takes place at the TCR beta region, TCR delta region and TCR alpha region of TCR loci. The gene rearrangement leads to the specific shape of the TCR, which is specific to an antigen. These T cells are known as a double positive T cells as they express CD4 and CD8 glycoproteins on the surface of the cell. These double positive T cells are then positively selected within the thymus upon their ability to bind to self-peptide via MHC class I and MHC class II binding (Anderson et al., 2005). To survive and further differentiate into a naïve T cell, double positive T cells must only bind to MHC class I and class II molecules weakly. If the interaction strength is correct, double positive T cells that interact with MHC class I molecules will mature into CD8+ T cells and cells that interact with MHC class II molecules will mature into CD4+ T cells (Anderson et al., 2002).

T cell maturation and development takes place within the thymus. During thymic development there is development of T cell receptor excision circles (TRECs). These are bi-products of TCR gene rearrangements similar to KRECs. During the maturation process of the TCR αβ receptor, the heavy and light chains undergo genomic rearrangement to form a functional receptor. TCR α (TCRA) genes contain the TCR variable (TCRV) and TCR joining (TCRJ) joining segments. The TCRA segment is excised through the gene rearrangement and forms a stable circular loop of DNA known as TRECs. TRECs are found in high copy numbers in naïve T cells that are released into the periphery upon T cell maturation within the thymus. Respectively, as T cell proliferation takes place within the periphery, TRECs become diluted as T cells mature and differentiate into further subtypes (Gaballa et al., 2016, Mensen et al., 2013, Ribeiro and Perelson, 2007, Sottini et al., 2014). In HSCT and CBT studies, TREC quantification has been performed to measure thymic output. Furthermore, TRECs have been quantified in healthy individuals and copy numbers of TREC decrease with age due to thymic involution (Douek et al., 1998, Gaballa et al., 2016, Junge et al., 2007, Kimmig et al., 2002, Kohler and Thiel, 2009, Kohler et al., 2005, Ribeiro and Perelson, 2007, Sottini et al., 2014).

Naïve T cells and recent thymic emigrants (RTEs), which emigrate from the thymus, contain high copy numbers of TRECs. CD31 is a surface marker expressed by T cells upon egression from the thymus (Kohler and Thiel, 2009). Additionally, CD31 (PECAM-1) is a member of the Ig superfamily and is a transmembrane surface marker expressed on a number of cells such as T cells, mast cells, NK cells, platelets and granulocytes (Newman, 1997). Kohler *et al*. has showed that CD31+ T cells contain high copy numbers of TRECs. This suggests that CD31+ T cells are naïve and are recently released from the thymus. In a study conducted by Komanduri *et al.* there are low absolute counts of RTEs and low copy numbers of TRECs for up to 365 days post-transplant. This suggests that thymic recovery is delayed in CBT patients (Komanduri et al., 2007).

1.12.6 Overall survival after cord blood transplant

Double CBT patients have a similar overall survival compared to patients transplanted with alternative donor sources such as BM, mPB and haploidentical transplant. This has been particularly seen in patients conditioned via myeloablative conditioning regimens (Brunstein et al., 2010, Liu et al., 2014, Takahashi et al., 2007). Respectively, RIC regimens also demonstrated that the overall survival of patients is similar to patients transplanted with BM and mPB and haploidentical graft sources (Brunstein et al., 2012, Chen et al., 2012, Majhail et al., 2008, Weisdorf et al., 2014, Malard et al., 2015). It has also been shown that patients infused with dCBU have similar disease free survival and overall survival compared to matched unrelated donor grafts (Rocha et al., 2004). This demonstrates that the overall survival of CBT patients is similar to the survival observed for a variety of graft sources used for transplantation. However, discrepancies may occur between studies, as there are patients within the respective studies with varying severity of disease. The survival time may differ between patients due to the inclusion of patients with myeloid and lymphoid malignancies.

1.12.7 Factors that affect immune reconstitution in cord blood transplant

Factors that affect immune reconstitution include: the type and intensity of conditioning regimens, the use of immunosuppressive therapy, GvHD and age of the recipient. These have been detailed in Table1.3.

Factor	Effect on immune reconstitution	Reference
Conditioning	- Presence of chemotherapeutic agents post-conditioning that could delay engraftment - Chemotherapeutic agents delay the maturation of T and B-cell subsets required for adaptive immune responses	(Brunstein et al., 2007)
Immunosuppressive therapy	- T-cell proliferation is inhibited by immunosuppressive drugs such as Ciclosporine A (CsA) and Mycophenolate mofetil (MMF) - ATG and Alemtuzamab (CAMPATH) deplete T cells	(Solomon et al., 2003, Storb et al., 1986, Mohty et al., 2004) (Pascal et al., 2015, Roll et al., 2015, Lowdell et al., 1998)
GvHD	- Induces thymic damage - Poor thymic function develops and results in exacerbated maturation of overactive T cells, which cause damage to the host - Causes delay in the B and T cell reconstitution and reductions of lymphocytic numbers takes place - aGvHD – strong inflammatory components causing tissue damage - cGvHD – autoimmune like features and fibrotic features	(Wu et al., 2013, Snover, 1984, MacMillan et al., 2009, Clave et al., 2009, Billingham, 1966)
Age of Recipient	- Patients between the ages of one to 24 have a greater rate of thymopoeisis compared to older patients. - Older patients have a reduced thymic homeostatic proliferation, which results in reduced thymic reconstitution.	(Mackall et al., 1995, Komanduri et al., 2007)

Table 1.3 Factors affecting immune reconstitution in CBT recipients

1.12.8 Immune reconstitution in cord blood transplant compared to other graft sources

Compared to other graft sources, CBT can be performed in a short period of time (Barker et al., 2002). However, due to the naivety of the immune cell content and lower TNC content, compared to other graft sources, CBT recipients have slower myeloid and platelet recovery. This increases the graft incidence of graft failure and non-relapse mortality in CBT patients compared to other graft sources (Brunstein et al., 2010, Jacobson et al., 2012).

Neutrophil engraftment takes place in the majority of CBT patients and can take place between 5-21 days (Laughlin et al., 2004, Rocha et al., 2004). Additionally, CB cells have a high proliferative capacity. However, the slower time to engraftment could be due to lower TNCs and fewer CD34+ cells within CB grafts compared to BM and mPB (Eapen et al., 2010, Atsuta et al., 2009). To overcome this two CB units have been used, which increases the TNC and CD34+ cells infused into patients (Barker et al., 2005). However, this does not enhance the time to engraftment in dUCBT patients compared to sUCBT recipients (Scaradavou et al., 2013, Verneris et al., 2009).

CBT patients conditioned with reduced intensity protocols have a faster neutrophil engraftment time compared to CBT patients conditioned with myeloablative protocols and recipients of BM and mPB (Oran et al., 2011, Chen et al., 2012, Sandhu et al., 2016). However, there is a greater risk of developing graft failure in RIC transplants, as the residual disease may not be completely cleared post-conditioning (Brunstein et al., 2012, Weisdorf et al., 2014).

1.12.8.1 NK cell reconstitution in cord blood transplant patients compared to other graft sources

NK cells are one of the first lymphocytes to reconstitute irrespective of the graft source. The kinetics of NK cell reconstitution is similar in CBT patients compared to mPB and BM recipients, occurring within the first month post-

transplant (Ottinger et al., 1996, Buhlmann et al., 2011, Shenoy et al., 1999, Jacobson et al., 2012, Brahmi et al., 2001). The reconstitution of NK cells occurs through the differentiation of progenitor NK cells. NK cells are of an immature $CD56^{bright}$ phenotype for several months and this shifts towards an increase in the $CD56^{dim}$ NK cell phenotype between three to six months post-transplant (Cooley et al., 2005, Beziat et al., 2009, Nguyen et al., 2005, Nguyen et al., 2008).

1.12.8.2 B cell reconstitution in cord blood transplant patients compared to other graft sources

B cells are also one of the first lymphocyte subsets to reconstitute within CBT patients. As shown in CBT studies, B cell reconstitution occurs within 180 days post-CBT. Comparatively, this is faster compared to BM and mPB recipients, where B cell reconstitution takes place between 180 and 365 days post-transplant (Avanzini et al., 2005, Lev et al., 2012, Marie-Cardine et al., 2008, Nakatani et al., 2014, Storek et al., 1993, Storek et al., 2001b). Furthermore, CBT patients reach the B cell absolute healthy adult range in a median time of 180 days, post-transplant (Nakatani et al., 2014). Moreover, B cells in CBT recipients produce higher amounts of B cell activating factor (BAFF) compared to mPB recipients, which promotes B cell survival (Jacobson et al., 2012). This suggests B cells could have a better survival in CBT recipients compared to mPB recipients.

1.12.8.3 T cell reconstitution in cord blood transplant patients compared to other graft sources

Studies comparing CBT to BMT and mPB transplant show that the absolute levels of CD3+, CD4+ and CD8+ T cells are lower in CBT recipients. Furthermore, T cell reconstitution is delayed for up to two years post-transplant and in some studies for up to five years post-transplant (Jacobson et al., 2012, Kanda et al., 2012, Beaudette-Zlatanova et al., 2013, Bejanyan et al., 2016, Mehta and Rezvani, 2016). Moreover, CBUs contain a higher proportion of

naïve T cells compared to other graft sources. However, several studies have shown that there are fewer circulating naïve T cells in CBT patients compared to BM and mPB patients (Kanda et al., 2012, Jacobson et al., 2012).

Both thymic dependent and thymic independent T cell reconstitution is delayed in CBT patients compared to BM and mPB recipients. Thymic output is delayed with lower TREC copy numbers and low absolute counts of RTEs in CBT recipients compared to BM and mPB recipients (Komanduri et al., 2007). In CBT patients, thymic independent reconstitution is skewed and patients have increased numbers of central memory and effector memory T cells post-transplant. However, the number of central memory and effector memory T cells are lower in CBT patients compared to BM and mPB recipients (Komanduri et al., 2007). Additionally, the reconstitution of Tregs in CBT patients is also delayed compared to mPB patients. The numbers of Tregs are lower in CBT patients up to 180 days post-transplant compared to mPB recipients (Jacobson et al., 2012).

1.13 Aims of the Project

Delayed immune reconstitution is a barrier towards successful CBT. Studies show that immune reconstitution is a major issue in HSCT and specifically in CBT (Komanduri et al., 2007, Jacobson et al., 2012). The delay in immune reconstitution leads to an increased risk of post-transplant complications such as relapse and increased risk of infection (Chakrabarti et al., 2002b, Chakrabarti et al., 2002a, Lamba et al., 2004).

Mapping the kinetics of immune cell reconstitution in CBT patients requires the investigation of a broad set of immune cells that play a fundamental role in innate and adaptive immune responses. The aim of this study is to define the kinetics and diversity of major immune cell subsets such as NK cells, B cells, T cells and their respective subpopulations, which will enhance our understanding of the immune cells that could influence post-transplant outcomes.

NK cells reconstitute within the first month post-CBT (Small et al., 1999, Jacobson et al., 2012, Brahmi et al., 2001). Furthermore, NK cells in CBT patients mediate GvL responses post-transplant (Beziat et al., 2011, Beziat et al., 2009). However, limited data exists on the activation of NK cells and their cytolytic capacity post-CBT. Therefore, the aim is to elucidate the functionality of NK cells in CBT patients and further analyse whether NK cells have a cytotoxic capacity against a malignant cell line.

B cells reconstitute within 180 days post-CBT (Nakatani et al., 2014), as a crucial component of adaptive immunity, B cells are required for the development of long-term immunity via the production of antigen specific antibodies. In CBT studies, the kinetics of B cell reconstitution has been focused on limited subsets of B cells such as transitional and mature B cell subsets (Cuss et al., 2006, Malaspina et al., 2007, Marie-Cardine et al., 2008, Sims et al., 2005). With this in mind, it is crucial to understand whether B cell developmental stages take place in CBT patients. This could provide an insight into the developmental stages involved in reconstituting B cell subsets and reconstitution of long-term immunity. Therefore, the aim of this current study is

to investigate the kinetics and diversity of B cell subsets in CBT patients with a further aim of elucidating whether there is reconstitution of developmental stages in B cell maturation.

Additionally, T cells are also a key cellular component of adaptive immune responses. Rapid reconstitution of T cells and expansion of T cell subsets in CBT patients has been associated with development of GvHD (Sakoda et al., 2007). T cells are further required post-transplant to reduce the incidence of relapse and for an optimised GvL response (Barrett, 2008). T cell reconstitution differs compared to NK cells and B cells and is delayed in CBT patients (Komanduri et al., 2007, Jacobson et al., 2012). Thymic dependent reconstitution has specifically been demonstrated as delayed in CBT patients (Gaballa et al., 2016, Kimmig et al., 2002, Sottini et al., 2014). However, thymic independent reconstitution in CBT patients includes the peripheral expansion of effector and central memory T cells, with reduced levels of naïve and RTEs (Komanduri et al., 2007). With this in mind, limited studies demonstrate a comprehensive analysis of thymic dependent and thymic independent reconstitution in CBT patients. Therefore, further work is required to better understand the reconstitution of T cell subsets in CBT patients.

Comprehensive studies are required to better understand the immune kinetics and diversity of reconstituting cell subsets in CBT patients. So far, published data demonstrates the reconstitution patterns of specific cell subsets in CBT patients. However, the patterns of reconstitution have not been broadly investigated in CBT patients within the UK. Herein, it highlights the importance of studying immune reconstitution in CBT recipients, as it will provide a better understanding of immune cell recovery post-transplant. The current study is the first clinical study within the UK to investigate the kinetics and diversity of reconstituting immune cell subsets in CBT patients.

The specific aims for this thesis were as follows:

Chapter 3 – To investigate the kinetics and diversity of recovering immune cell subsets in CBT patients.

Chapter 4 – To investigate NK cell functionality in CBT patients.

Chapter 5 – To investigate the kinetics and diversity of B cell reconstitution and whether reconstitution of B cells impacts the overall survival in CBT patients.

Chapter 6 – To investigate the kinetics and diversity of T cell reconstitution in CBT patients.

Chapter 2 : Materials and Methods

2.1 Blood

2.1.1 Umbilical cord blood

All CB units were obtained from the Anthony Nolan Cord Blood Bank, Nottingham. CB was obtained under prior informed consent and approval by the ethical committee (NREC Reference no 10/H0405/27). All samples were processed within 24 hours upon delivery to the Anthony Nolan Research Institute.

2.1.2 Peripheral blood

Healthy adult peripheral blood (PB) blood was obtained from healthy volunteers with informed consent.

2.1.3 Patient blood

Patients undergoing CBT as a treatment for a variety of haematological malignancies and disorders were recruited at the respective hospitals:

1. Beatson West of Scotland Cancer Centre, Glasgow
2. Bristol Royal Infirmary
3. Christies NHS foundation Trust, Leeds
4. Freeman Hospital, Newcastle
5. Heart of England NHS Foundation Trust, Birmingham
6. Kings College Hospital, London
7. Manchester Royal Infirmary
8. Queen Elizabeth Hospital, Birmingham
9. Royal Hallamshire Hospital

10. Royal Marsden Hospital, Sutton
11. St. Bartholomew's Hospital, London
12. St James's Hospital, Leeds
13. UCLH, London

CBT Patients were recruited between January 2009 and October 2015. Patients provided informed consent before a blood sample was obtained. Patient blood samples were taken at the same time as routine hospital checks and patients did not require an additional vene-puncture. Blood samples were collected from patients at 28, 60, 100, 180, 365 and 720 days post-transplant. At the respective time points 40 mL of PB were obtained in transport media and 10 mL of PB in EDTA vacutainer. All samples were processed within 24 hours upon delivery to the Anthony Nolan Research Institute.

The Immune reconstitution study (IRES-CBT, REC number: 09/H0706/35) was set up in 2009 and has been funded by the UK Stem Cell Foundation and Anthony Nolan. Patient samples were initially collected by Dr Sameer Tulpule Patient sample collection and processing was followed up by Dr Damini Tewari. I then started patient sample collection and processing on May 1st 2012. At the respective time points the number of samples processed by myself are listed in Table 2.1.

Day	28	60	100	180	365	720
Samples Processed	38	38	38	25	26	14

Table 2.1 Number of samples processed by myself during the immune reconstitution study

2.2 Materials used for cellular studies

All the materials used in the cellular studies are stated in table 2.2.

Media/Reagents/Serum	Manufacturer	Country
β-mercaptoethanol (β-ME)	Life technologies	Paisley, UK
Bovine Serum Albumin	Sigma	Poole, UK
Dextran 40	Fresenius Kabi	Barcelona, Spain
DNAase – 1	Merck K GaA	Darmstadt, Germany
Dimethyl sulphoxide (DMSO)	Sigma	Poole, UK
Ethylenediaminetetraacetic acid (EDTA) 0.5M ultra pure pH8.0	Life Technologies	Paisley, UK
Fetal Calf Serum (FCS)	Lonza	Verviers, Belgium
Fixation/ Permeabilization buffer	eBiosciences	Hatfield, UK
FACS Lysis Solution	BD Pharmingen	Oxford, UK
Lympholyte-H	VWR	Leicestershire, UK
Magnesium Chloride ($MgCl_2$)	Sigma	Poole, UK
Phosphate Saline Buffer (PBS) 10X	Lonza	Verviers, Belgium
Penicillin and Streptomycin (Pen/strep)	Lonza	Verviers, Belgium
Pharm Lyse	BD Pharmingen	Oxford, UK
Proteinase K	Sigma	Poole, UK
Roswell Park Memorial Institute media with L-glutamine 1640 (RPMI-1640)	Lonza	Verviers, Belgium
Sterile Water	Baxter	Zurich, Switzerland
Trisodium Citrate	Sigma	Poole, UK
Tryphan Blue (0.4%)	Sigma	Poole, UK

Table 2.2 Media, reagents and serum used in this study.

All the materials used during cellular studies are listed in Table 2.3.

Material	Manufacturer	Country
1 mL, 5 mL, 10 mL and 50 mL syringes	BD Plastipack	Madrid, Spain
5 mL polypropylene round bottom tubes	BD Pharmingen	Erembodgen, Belgium
Cryotube Vials	Nunc	Roskilde, Denmark
CoolCell Cell Freeze Containers	Sigma-Aldrich	St. Louis, Missouri, United States
EDTA - 10.0 mL BD Vacutainer® plastic EDTA tube. Lavender BD Hemogard™ closure	BD Pharmingen	Erembodgen Belgium
Falcon Tube (15 mL, 50 mL)	Starsted	Numbrecht, Germany
Minidstart Sterile Filter	Sartorius Stedium Biotech	Munich, Germany
Partec CellTrics 30 micron filters	Partec	Germany
Pasteur Pipette	Thermo Fisher Scientific	Loughborough, UK
Serological pipette (5 mL, 10 mL, 25 mL)	Starstedt	Numbrecht, Germany
Tissue Culture Flask (25, 75 and 150 cm^2)	Starstedt	Numbrecht, Germany
TruCount Tubes	BD	Oxford, UK
U-bottom 96-well plate	Starstedt	Numbrecht, Germany
V-bottom 96-well plate	Starstedt	Numbrecht, Germany

Table 2.3 List of materials used during cellular studies.

2.2.1 Buffers

All the buffers used for cell staining, isolation and washing are listed in Table 2.4.

Buffer	Components
Cell Thawing Buffer	RPMI 1640 + 20% FCS
Dextran Thawing Buffer	5% FCS, 0.63% Trisodium Citrate, 5 mM $MgCl_2$, 1000 IU/mL DNase-1 in Dextran 40
FACS buffer	1X PBS and 10%V/V FBS
Lysing Buffer	10X BD FACS lysis solution diluted to 1X with distilled water
MACS buffer	1X PBS, 1%w/v BSA and 2mM EDTA
PBS	10X PBS diluted to 1X with distilled water

Table 2.4 List of Buffers

2.2.2 Media

All the media used for cellular studies are listed in Table 2.5.

Medium	Components
Culture medium	RPMI 1640, 5μM 2 β-ME, 1% Pen-Strep and 10% v/v FCS
Freeze Media	FCS, 10% DMSO
Transport Medium	RPMI 1640, 33% v=w/v Tri-sodium citrate, 5μM 2 β-ME

Table 2.5 List of Media

2.2.3 Kits

All the commercial kits used for cellular isolation and DNA/RNA extraction are listed in Table 2.6.

Kit	Manufacturer
BD Cytofix/Cytoperm Kit	BD, Frembodgen, Belgium
FoxP3 Transcription Factor Fixation/Permeabilization Kit	eBiosciences, Hatfield, UK
NK Cell Isolation Kit	Miltenyi Biotec, Germany
PKH67 Green Flourescent Cell Linker Kit for General Cell membrane Labelling	Sigma, Poole, UK
PureYield Plasmid Miniprep Kit	Promega, Wisconsin, USA
RNeasy Mini kit	Qiagen, Germany

Table 2.6 List of Kits

2.2.4 Antibodies

All the antibodies used in immune reconstitution immunophenotyping are listed in Table 2.7. Furthermore, all antibodies used for immunophenotyping in immune function analysis are listed in Table 2.8.

Surface Marker	Company	Catalogue Number	Fluorochrome	Dilution	Clone	Isotype
CCR7	BD	560765	PE	1/20	150503	Mouse IgG2a
CD3	BD	345766	PerCP	1/10	SK7	Mouse BALB/c IgG1, κ
CD3	BD	555335	APC	1/10	UCHT1	Mouse IgG1, κ
CD4	BD	555346	FITC	1/100	RPA-T4	Mouse IgG1, κ
CD4	BD	345770	PerCP	1/100	SK3	Mouse IgG1, κ
CD8	BD	555634	FITC	1/10	HIT8a	Mouse IgG1, κ
CD10	BD	555375	PE	1/10	HI10a	Mouse IgG1, κ
CD14	BD	555397	FITC	1/100	M5E2	Mouse IgG1, κ
CD16	BD	555406	FITC	1/5	3G8	Mouse IgG1, κ
CD19	BD	345778	PerCP	1/20	4G7	Mouse IgG2a, κ
CD19	BD	555415	APC	1/20	HIB19	Mouse IgG1, κ
CD21	BD	559867	APC	1/10	B-ly4	Mouse IgG1, κ
CD24	BD	555427	FITC	1/10	ML5	Mouse IgG1, κ
CD25	Biolegend	302625	PerCP-Cy5.5	1/20	BC96	Mouse IgG1, κ
CD27	BD	555440	FITC	1/10	M-T271	Mouse IgG2a, κ
CD31	BD	555446	PE	1/50	WM59	Mouse IgG1, κ
CD38	BD	555460	PE	1/10	HIT2	Mouse IgG1, κ
CD45	BD	332784	PerCP-Cy5.5	1/50	2D1	Mouse IgG1, κ
CD45	BD	555485	PerCP	1/10	H130	Mouse IgG1, κ
CD45RA	BD	550855	APC	1/100	HI100	Mouse IgG1, κ
CD56	BD	555516	PE	1/10	B159	IgG1, κ
CD127	BD	557938	PE	1/50	HIL-7R-M21	Mouse IgG1, κ

Table 2.7 Cell surface staining antibodies for immune reconstitution.

Surface Marker	Company	Catalogue Number	Fluorochrome	Dilution	Clone	Isotype
CD3	BD	557851	PE-Cy7	1/10	SK7	Mouse IgG1, κ
CD3	BD	561027	Alexa Flour 700	1/50	UCHT1	Mouse BALB/c IgG_1, κ
CD4	BD	555349	APC	1/20	RPA-T4	Mouse IgG_1, κ
CD8	BD	565310	PerCP-Cy5.5	1/20	SK1	Mouse BALB/c IgG_1, κ
CD8	BD	555634	FITC	1/20	HIT8a	Mouse IgG_1, κ
CD14	BD	555399	APC	1/25	M5E2	Mouse IgG_{2a}, κ
CD16	BD	555406	FITC	1/20	3G8	Mouse BALB/c x DBA/2
CD16	Biolegend	302011	APC	1/50	3G8	Mouse IgG1, κ
TCR -αβ	BD	555547	FITC	1/10	T10B9.1A-31	Mouse BALB/c IgM, κ
TCR- γδ	BD	555717	PE	1/20	B1	Mouse IgG_1, κ
CD56	BD	560842	PerCP-Cy5.5	1/10	B159	Mouse IgG_1, κ
CD56	BD	557747	PE-Cy7	1/20	B159	Mouse IgG_1, κ
CD57	BD	562488	PE-CF594	1/10	NK-1	Mouse IgM, κ
CD25	BD	341011	PE	1/25	M-A251	Mouse BALB/c IgG_1, κ
CD25	BD	555434	APC	1/20	M-A251	Mouse BALB/c IgG_1, κ
CD69	BD	557756	APC-Cy7	1/10	FN50	Mouse IgG_1, κ
HLA-DR	BD	560651	PE-Cy7	1/10	G46-6	Mouse IgG_{2a}, κ
NKG2A	R&D Systems	FAB1059A	APC	1/10	131411	Mouse IgG_{2A}
NKG2C	Biolegend	FAB138P	PE	1/10	134591	Mouse IgG_1
IFN-γ	BD	552887	PE	1/100	B27	Mouse IgG_1, κ
CD127	BD	557938	PE	1/100	HIL-7R-M21	Mouse IgG_1, κ
FoxP3	eBioscience	45-4776-42	PerCP-Cy5.5	1/80	PCH101	Rat IgG2a

Table 2.8 Antibodies used for immunophenotyping for functional analysis

2.2.5 Materials used for molecular work

Media/Reagents/Serum	Manufacturer	Country
ABI BigDye Terminators V3.0 ready reaction mix	Applied Biosystems	California, USA
Absolute Ethanol	VWR	Leicestershire, UK
BigDye reaction buffer	Applied Biosystems	California, UK
EcoRI restriction enzyme	Sigma	Poole, UK
Ethidium Bromide (EtBr)	Sigma	Poole, UK
EDTA 0.5M Ultra pure pH8.0	Life Technologies	Paisley, UK
Glycerol	VWR	Leicestershire, UK
HEPES buffer (pH7.4) 10mM	Sigma	Poole, UK
HiDI Formamide solution	Applied Biosystems	California, UK
Hyperladder IV	Bioline	London, UK
Kanamycin	Sigma	Poole, UK
LE Agarose	Invitrogen	Paisley, UK
$MgCl_2$ 50 mM	Bioline	London, UK
Oneshot TOP10 Component cells	Invitrogen	Carlsbald, USA
Proteinase K	Sigma	Poole, UK
Sodium Chloride (NaCl)	Sigma	Poole, UK
Sodium Dodecyl Sulphate (SDS)	Sigma	Poole, UK
Sterile Water	Baxter	Zurich, Switzerland
Tris	Sigma	Poole, UK
Tris-Boric Acid EDTA (TBE)	Sigma	Poole, UK
TaqMan Universal Master Mix, with UNG	Life Technologies	Paisley, UK
Xho I restriction enzyme	Sigma	Poole, UK

Table 2.9 List of Media, reagents and serum used for molecular work.

All the materials used in molecular studies are listed in Table 2.10.

Materials	Manufacturer	Country
Hard-shell PCR plates 96-well, thin wall	Bio-Rad	Oxford, UK
PCR Plate Sealer	Bio-Rad	Oxford, UK
Whatman filter paper	Whatman Inc	New Jersey, USA

Table 2.10 Materials used for molecular studies

2.2.6 Instruments and software

All the instruments and software used for molecular biology work are listed in Table 2.11.

Instrument	Software
ABI GA-3730xl genetic analyser (Applied Biosystems, Foster City, California, USA).	Data Collection software version 2.0,
Bio-Rad CFX96 Real-Time System	Bio-Rad CFX Manager software version 3.1
NanoDrop – 1000 Spectrophotometer (US)	NonoDrop-1000 version 3.3.1

Table 2.11 Instruments and software used for molecular studies

2.3 Methods

2.3.1 Cellular biology

All cellular work was conducted in a class II laminar flow tissue culture cabinet (Astec Micro, UK) using aseptic techniques and sterile laboratory consumables.

2.3.2 Mononuclear cell isolation

Patient and healthy donor peripheral blood mononuclear cells (PBMC) or Cord blood mononuclear cells (CBMC) samples in transport media were separated via density gradient centrifugation using Lympholyte-H at 2200 rpm for 22 minutes with the break off at 500 rpm. The mononuclear layer was collected and washed twice; the first wash was undertaken at 1800 rpm for 10 minutes and then 1600 rpm for 5 minutes. The PBMC pellet was re-suspended for cell enumeration however for the CBMC pellet required further lysis to remove red blood cells.

2.3.3 Red blood cell lysis

The amount of red blood cells harvested from the mononuclear layer was higher in CB than in PB. Therefore a lysis step was required for 8 minutes at room temperature with 1X lysing buffer for CBMC. Cells were then washed with RPMI 1640 and centrifuged at 1800rpm for 10 minutes.

2.3.4 Cell enumeration and viability

Cell number and viability measurements were performed using two different exclusion dye methods: Trypan Blue to determine viability and Turk's count to determine the number of nucleated cells. For both methods, the solution of Trypan Blue or Turk's was in a 1:1 ratio with cells within a 0.1mm haemocytometer counting chamber (Hawkley, UK). Viable cells were counted under a phase contrast DM LB Microscope (Leica, Germany).

2.3.5 Cell cryopreservation

For cryostorage, isolated cells were re-suspended in Freeze Media. Isolated mononuclear cells were stored in cryotube vials at a concentration of 10 x 10^6 cells and 5 x 10^6 cells in a 1 mL and 0.5 mL respective volume. The cells were stored at this concentration to ensure a balanced concentration allowing the cells to mix evenly within freezing medium. Cryovials containing the cell suspensions were placed into -70^{o}C within CoolCell Cell freezing containers. This allowed a liquid cooling rate of 1^{o}C per minute. After 24 hours the cells were transferred to liquid nitrogen for long-term storage.

2.3.6 Cell thawing

Healthy donor and patient PBMCs or CBMC samples were thawed in a 37^{o}C water bath (Fischer Scientific, UK) and mixed very gently with a cooled thawing mix of Dextran thawing buffer. Patient PBMCs and healthy control PBMCs were thawed in a cell thawing buffer and rested for 30 minutes before being centrifuged at 1600 rpm for 5 minutes. The cells were then resuspended in RPMI 1640.

2.3.7 Immunophenotyping for immune reconstitution

Immunophenotyping panels (Table 2.12) were established to identify cellular reconstitution in CBT patients.

Cell subtype	FITC	PE	PerCP	APC
T-cells/B-cells/Natural Killer Cells	CD16	CD56	CD3	CD45
Monocytes/B-cells	CD14	-	CD45	CD19
Regulatory T-cells	CD4	CD127	CD25	CD3
T-stages	CD4	CCR7	CD3	CD45RA
T stages	CD8	CCR7	CD3	CD45RA
B stages	CD24	CD38	CD4	CD19
B stages	CD27	CD10	CD19	CD21
Recent Thymic Emigrants	CD4	CD31	CD3	CD45RA
Recent Thymic Emigrants	CD8	CD31	CD3	CD45RA

Table 2.12 Fluorochrome staining panel for assessment of immune recovery and kinetics in CBT patients

The immunophenotyping panel was established in 2009 by Dr Sameer Tulpule and the panel set up was used throughout the immune reconstitution study to ensure consistent analysis between patient samples. The immunophenotyping panel was established so that the following cellular subsets could be analysed within CBT patients.

1) T cells (CD3+)
2) CD14+ monocytes
3) CD19+ B-cells
4) CD45+ cells
5) Regulatory T cells (Tregs): CD3+ CD25+ CD25+ $CD127^{low}$
6) CD4 T-stages
   a) Naïve T cells - CD3+CD4+CD45RA+CCR7+
   b) Effector T cells - CD3+CD4+CD45RA+CCR7-
   c) Effector memory T cells - CD3+CD4+CD45RA-CCR7-
   d) Central memory T cells - CD3+CD4+CD45RA-CCR7+
7) CD8 T-stages
   a) Naïve T cells - CD3+CD8+CD45RA+CCR7+
   b) Effector T cells - CD3+CD8+CD45RA+CCR7-
   c) Effector memory T cells - CD3+CD8+CD45RA-CCR7-
   d) Central memory T cells - CD3+CD8+CD45RA-CCR7+
8) B-cells
   a) Transitional B cells – CD4-CD19+CD24+CD38+
   b) Mature B cells – CD4-CD19+CD24+CD38-
   c) Activated B cells - CD19+CD10-CD21+CD27+
   d) Memory B cells - CD19+CD10-CD21-CD27+
   e) Naive B cells - CD19+CD10+CD21+CD27-
9) CD4 recent thymic emigrants – CD3+CD4+CD31+CD45RA+
10) CD8 recent thymic emigrants – CD3+CD8+CD31+CD45RA+

2.3.8 Cell surface staining of whole blood for flow cytometry

The 10 mL EDTA patient blood sample was used for cell surface staining to identify the cell subsets listed in the Table 2.12. 50 µL of blood was aliquoted into ten 5 mL polystyrene round bottom tubes. 25 µL of the respective antibody master mix was added to each round bottom tube before being incubated at 4^{o}C for 20 minutes. The red blood cells were lysed using FACS lysing solution for 10 minutes at room temperature. Thereafter, the cells were centrifuged for 5 minutes at 1200 rpm. The supernatant was discarded and the cells were re-suspended in 1 mL of FACS buffer. The cells were centrifuged for 5 minutes at 1200 rpm. The supernatant was discarded and the cells were re-suspended in 400 µL of FACs buffer. The cells were acquired on the flow cytometer within 1 hour.

2.3.9 Measurement of absolute cell count numbers in whole blood using TruCount staining

Absolute cell numbers in each whole blood sample were measured within two hours of receiving the sample using TruCount^{TM} Tubes. Each TruCount^{TM} tube contains a pre-determined number of fluorescent beads and during the acquisition and analysis are gated to compare the cell frequency in each blood sample, which was stained with fluorochrome-labelled antibodies as listed in Table 2.7 to make up cocktails shown in table 2.12.

50 µl of whole blood was pipetted into each tube. Two separate tubes were used: one containing 25 µl CD16-FITC/CD56-PE/CD3-PerCP/CD45-APC labelled antibodies, the other containing 25 µl CD14-FITC/CD45-PerCP/CD19-APC labelled antibodies. Samples were then vortexed and incubated in the dark at room temperature for 15 minutes. Thereafter 450 µl of 1X FACS^{TM} Lysis solution was added to each tube and incubated in the dark at room temperature for 15 minutes. The samples were acquired on the flow cytometer within one hour.

2.3.10 Flow cytometry sample acquisition

Characterisation of the cell subsets was undertaken via flow cytometry using a FACSCalibur (BD, UK), FACSCanto II (BD, UK) and LSR Fortessa (BD, UK). Data analysis was undertaken via the use of FlowJo software (Treestar Inc., USA). Single stains and negative controls were used to specifically validate the cell populations within all samples acquired for flow cytometry analysis. Patient samples were initially processed using the NHSBT - Colindale facilities; this covered the time in which laboratory protocols could be set up at the Anthony Nolan Research Institute. At the NHSBT – Colindale the FACSCanto II (BD, UK) were used to acquire samples. Furthermore, at the Anthony Nolan Research Institute, the FACS Calibur (BD, UK) and the LSR Fortessa (BD, UK) were used to acquire samples.

2.3.11 Clinical data

Clinical data for each patient was obtained through the Eurocord Database. This data was collected at the respective transplant centres by clinical staff during the patient follow-ups and submitted to the Eurocord Database. (In this study, the median follow up of survivors was 25 months). The data was extracted from the Eurocord database and sent to myself at the Anthony Nolan Research Institute.

2.4 Characterisation of cellular subsets gating strategies for immune reconstitution

The following gating strategy shown in Figure 2.1 was used to analyse and quantify CD3, CD16, CD45 and CD56 Respectively, the following cellular subsets were quantified within healthy control and patient PB samples: CD56+ (NK-cells), CD3+ (T cells), $CD56^{bright}$ $CD16^{negative}$ and $CD56^{dim}$ $CD16^{positive}$ (NK cells) and CD45+ cells.

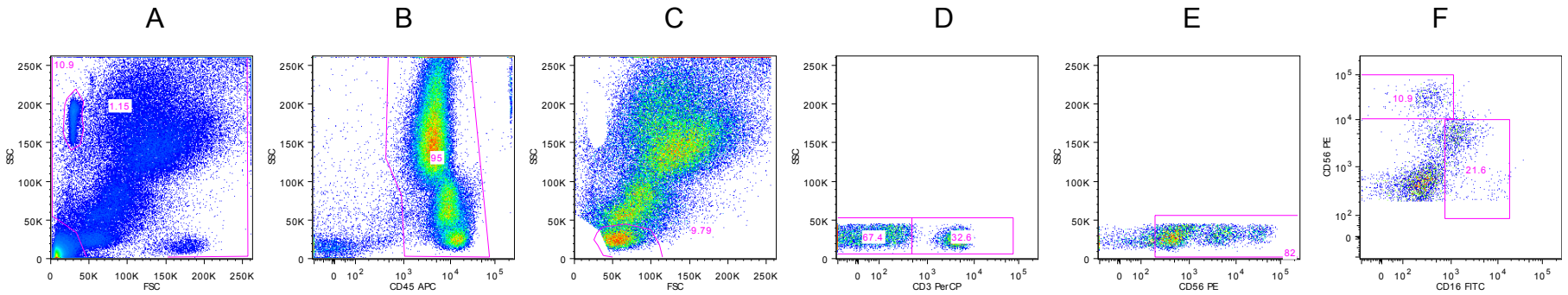


Figure 2.1 Gating strategy for absolute counts (Panel 1). Healthy control and patient PB samples were stained with monoclonal antibodies for surface expression of CD3, CD16, CD56 and CD45. (A) Gating of TruCount beads, (B) Gating of the CD45+ cells, (C) Gating of lymphocytes (D) Gating of CD3+ and CD3- (E) Gating of CD56+ cells, (F) Gating of $CD56^{dim}$ and $CD56^{bright}$ to identify NK cells.

The following gating strategy shown in Figure 2.2 was used to identify and quantify the following cell subsets in healthy control and patient PB samples; CD14+ (Monocytes), CD19+ (B cells) and CD45+ cells.

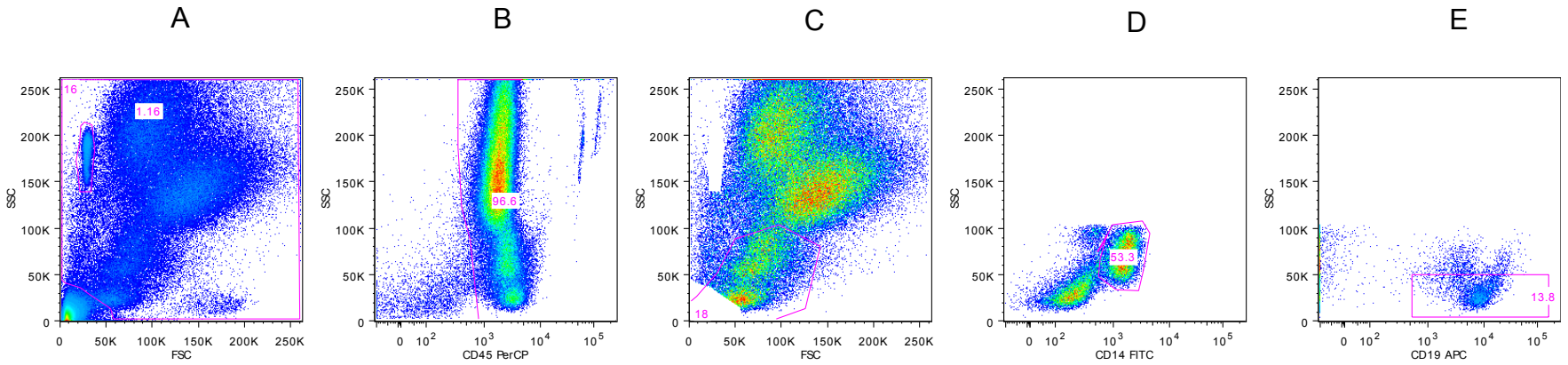


Figure 2.2 Gating strategy for absolute counts (Panel 2). Healthy control and patient PB samples were stained with monoclonal antibodies for surface expression of CD14, CD19 and CD45. (A) Gating of TruCount beads, (B) Gating of the CD45+ cells, (C) Gating of lymphocytic and monocytic cell populations, (D) Gating of CD14+ Monocytes from the CD45+ cells, (E) Gating of CD19+ B cells from the CD45+ cells.

The following gating strategy shown in Figure 2.3 was used to analyse and quantify Regulatory T cells (CD3+CD4+CD25+CD127lo) in healthy control and patient PB samples.

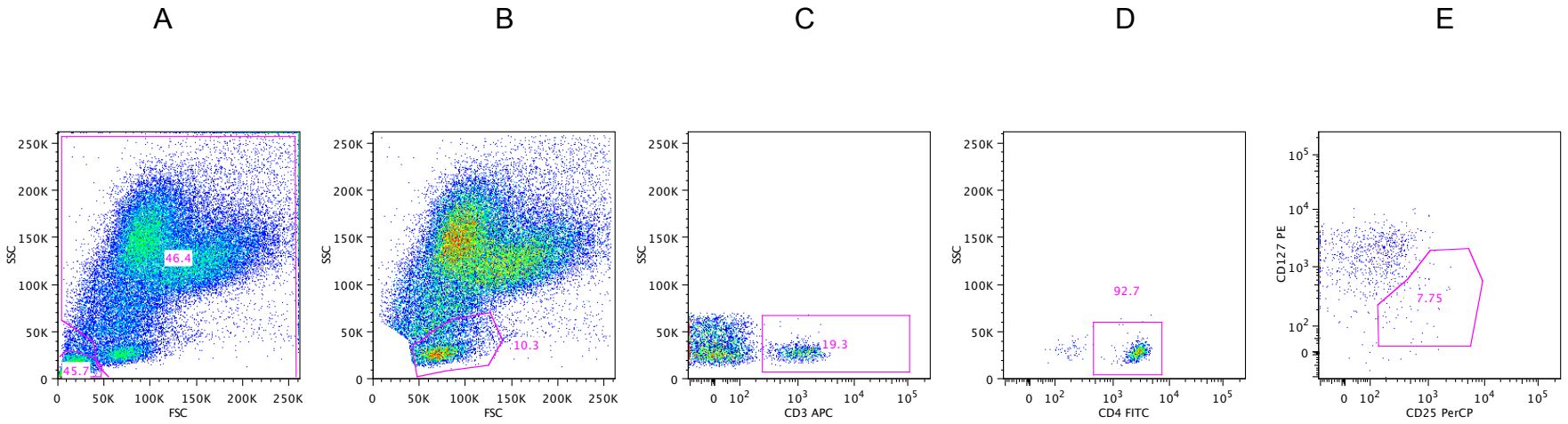


Figure 2.3 Gating of Regulatory T cells. Healthy control and patient PB samples were stained with monoclonal antibodies for surface expression of CD3, CD4, CD25 and CD127. (A) Gating of lymphocytes and exclusion of debris, (B) Gating of lymphocytes, (C) Gating of CD3+ T cells, (D) Gating of CD4+ T cells, (E) Gating of CD4+CD25+CD127^{lo} Regulatory T cells.

The following gating strategy shown in Figure 2.4 was used to identify and quantify CD4+ T stages within healthy control and patient PB samples. Respectively the following subsets were identified: naïve T cells (CD3+CD4+CD45RA+CCR7+), central memory T cells (CD3+CD4+CD45RA-CCR7+), effector T cells (CD3+CD4+CD45RA+CCR7-) and effector memory T cells (CD3+CD4+CD45RA-CCR7-).

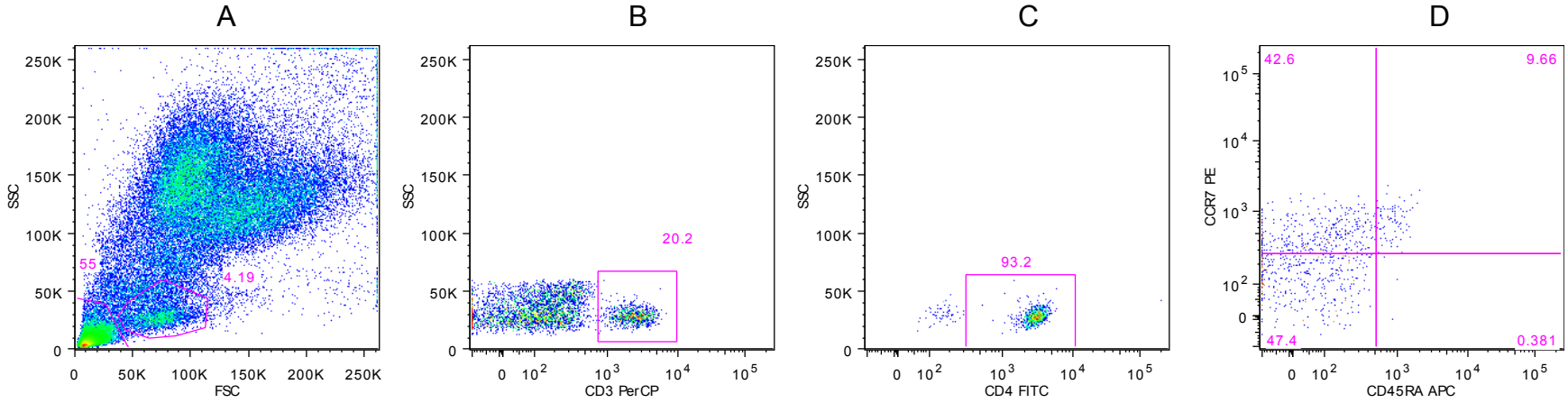


Figure 2.4 Gating strategy for CD4+ T stages. Healthy control and patient PB samples were stained with monoclonal antibodies for surface expression of CD3, CD4, CD45RA and CCR7. (A) Gating of lymphocytes and exclusion of debris, (B) Gating of CD3+ T cells, (C) Gating of CD4+ T cells, (D) Gating of CD45RA and CCR7 subsets to distinguish the following cell subsets: naïve T cells (CD3+CD4+CD45RA+CCR7+), central memory T cells (CD3+CD4+CD45RA-CCR7+), effector T cells (CD3+CD4+CD45RA+CCR7-) and effector memory T cells (CD3+CD4+CD45RA-CCR7-).

The following gating strategy shown in Figure 2.5 was used to identify and quantify CD8+ T stages within healthy control and patient PB samples. Respectively the following subsets were identified: naïve T-cells (CD3+CD8+CD45RA+CCR7+), central memory T cells (CD3+CD8+CD45RA-CCR7+), effector T cells (CD3+CD8+CD45RA+CCR7-) and effector memory T cells (CD3+CD8+CD45RA-CCR7-).

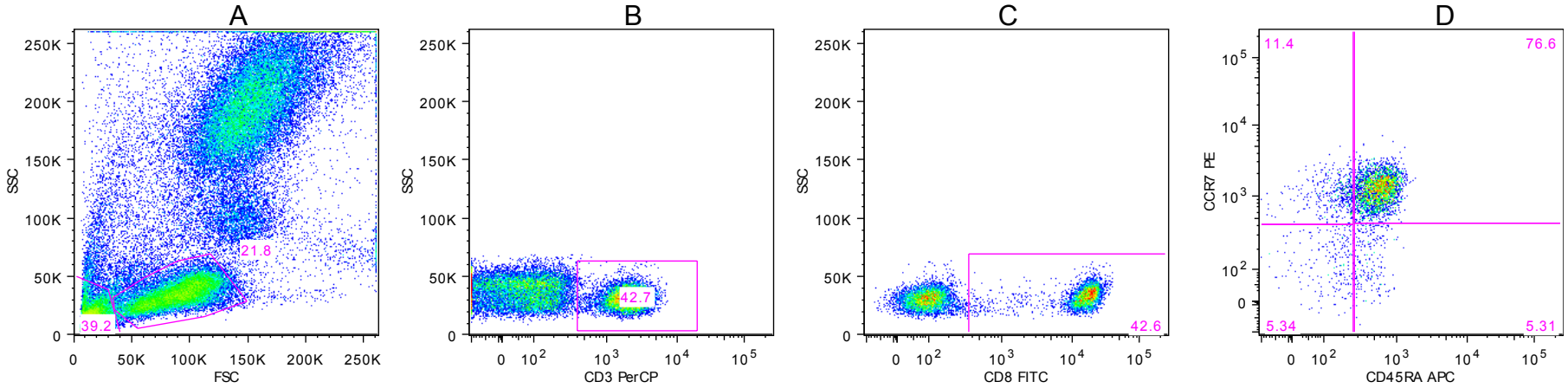


Figure 2.5 Gating strategy for CD8+ T stages. Healthy control and patient PB samples were stained with monoclonal antibodies for surface expression of CD3, CD8, CD45RA and CCR7. (A) Gating of lymphocytes and exclusion of debris, (B) Gating of CD3+ T cells, (C) Gating of CD8+ T cells, (D) Gating of CD45RA and CCR7 subsets to distinguish the following cell subsets: naïve T cells (CD3+CD8+CD45RA+CCR7+), central memory T cells (CD3+CD8+CD45RA-CCR7+), effector T cells (CD3+CD8+CD45RA+CCR7-) and effector memory T cells (CD3+CD8+CD45RA-CCR7-).

The following gating strategy shown in Figure 2.6 was used to identify and quantify mature and transitional B cells within healthy control and patient PB samples. Respectively the following subsets were identified: transitional (CD4-CD19+CD24+CD38+) and mature B cells (CD4-CD19+CD24+CD38-).

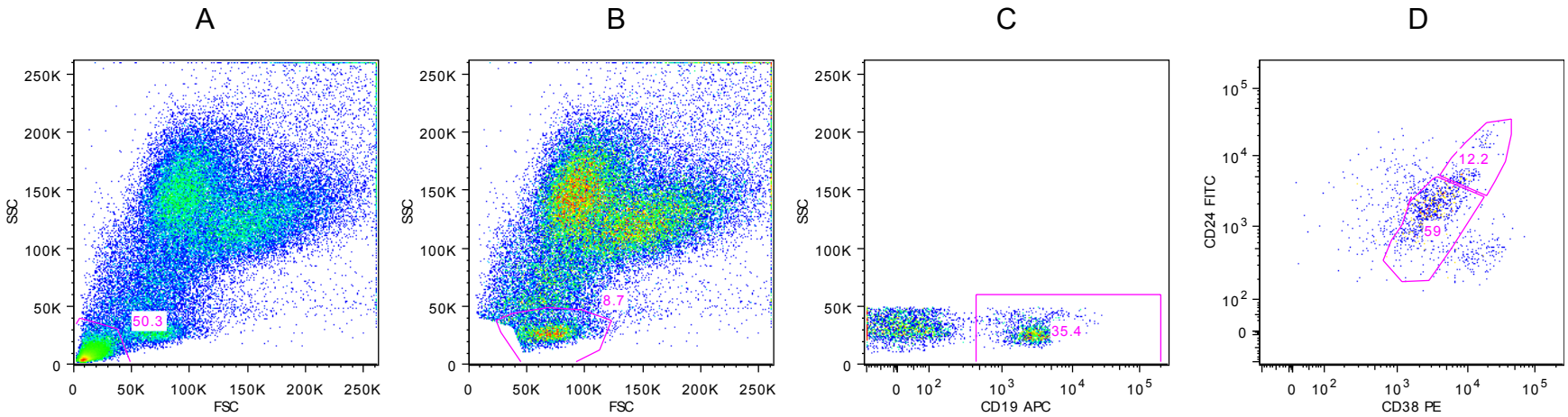


Figure 2.6 Gating strategy for Transitional and Mature B cells. Healthy control and patient PB samples were stained with monoclonal antibodies for surface expression of CD4, CD19, CD24 and CD38. (A) Gating of lymphocytes and exclusion of debris, (B) Gating of lymphocytes, (C) Gating of CD19+ B cells, (D) Gating of CD24 and CD38 subsets to distinguish the following cell subsets: transitional (CD4-CD19+CD24+CD38^{high}) and mature B cells (CD4-CD19+CD24+CD38^{low}).

The following gating strategy shown in Figure 2.7 was used to identify and quantify the following B cell subsets within healthy control and patient PB samples: activated B-cells (CD19+CD10-CD21+CD27+), memory B-cells (CD19+CD10-CD21-CD27+) and naive B-cells (CD19+CD10-CD21+CD27-).

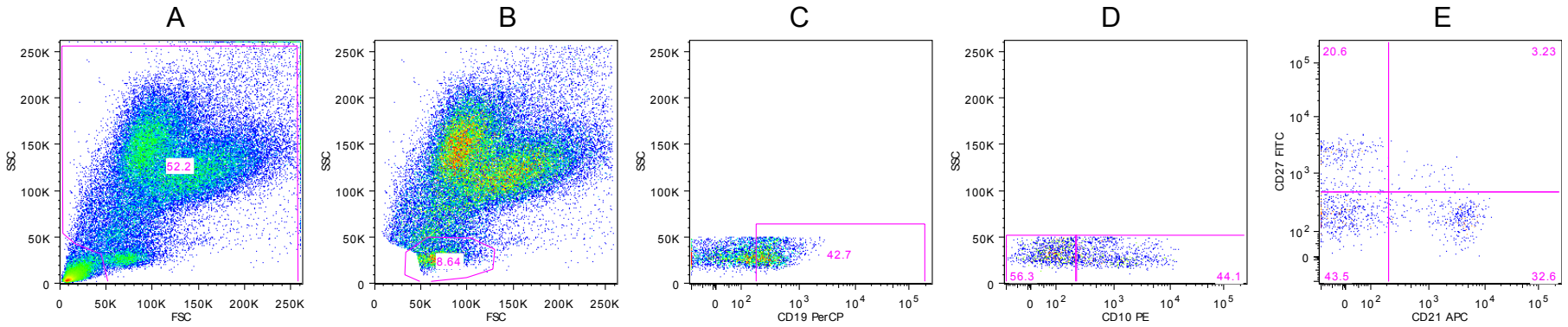


Figure 2.7 Gating strategy for Activated B cells, Memory B cells and Naive B cells. Healthy control and patient PB samples were stained with monoclonal antibodies for surface expression of CD10, CD19, CD21 and CD27 (A) Gating and exclusion of debris, (B) Gating of lymphocytes, (C) Gating of CD19+ B cells, (D) Gating of CD10+ and CD10- B cells (E) Gating of CD10- subsets: activated B cells (CD19+CD10-CD21+CD27+), memory B cells (CD19+CD10-CD21-CD27+) and naive B cells (CD19+CD10-CD21+CD27-**).**

The following gating strategy shown in Figure 2.8 was used to identify and quantify CD4+ T cell recent thymic emigrants (CD3+CD4+CD31+CD45RA+) within healthy control and patient PB samples.

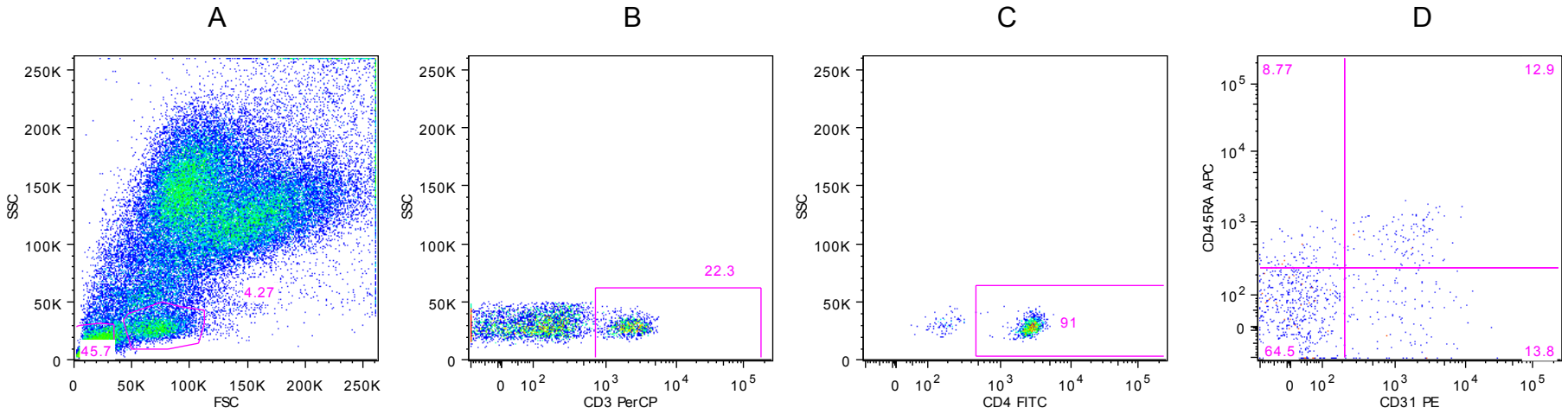


Figure 2.8 Gating Strategy for CD4+ T cell recent thymic emigrants. Healthy control and patient PB samples were stained with monoclonal antibodies for surface expression of CD3, CD4, CD31 and CD45RA (A) Gating of lymphocytes and exclusion of debris, (B) Gating of CD3+ T cells, (C) Gating of CD4+ T cells, (D) Gating of CD45RA and CD31 to distinguish CD4+ recent thymic emigrants (CD3+CD4+CD31+CD45RA+).

The following gating strategy shown in Figure 2.9 was used to identify and quantify CD8+ T cell recent thymic emigrants (CD3+CD8+CD31+CD45RA+) within healthy control and patient PB samples.

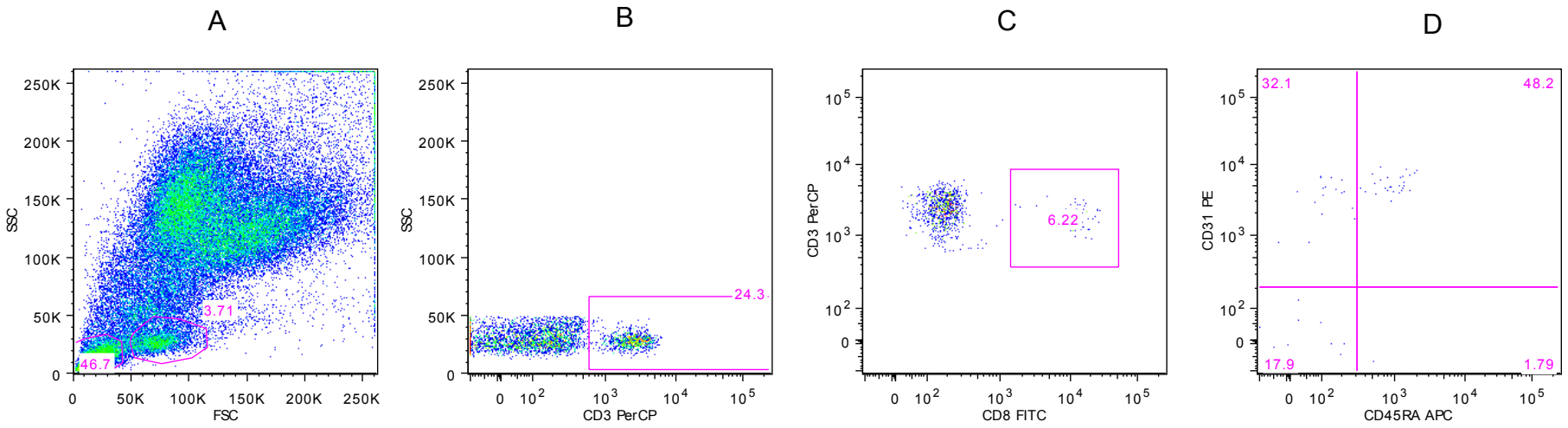


Figure 2.9 Gating strategy for CD8+ T cell recent thymic emigrants. Healthy control and patient PB samples were stained with monoclonal antibodies for surface expression of CD3, CD8, CD31 and CD45RA. (A) Gating of lymphocytes and exclusion of debris, (B) Gating of CD3+ T cells, (C) Gating of CD8+ T cells, (D) Gating of CD45RA and CD31 to distinguish CD8+ recent thymic emigrants (CD3+CD8+CD31+CD45RA+).

2.5 Patient flow cytometry database

Flow cytometric data was recorded onto a database constructed by myself to handle and manage the subset data. FileMaker Pro software was used to construct the database and different layouts were produced so that flow cytometric data could be stored for the following cellular subsets:

- Figure 2.10 – Database main page
- Figure 2.11 - Absolute counts 1 for CD3+ T cells, CD16, CD45+ cells, CD56+ NK cells and $CD56^{dim}$ $CD16^{positive}$ NK cells
- Figure 2.12 - Absolute counts 2 for CD14+ monocytes, CD19+ B cells and CD45+ cells
- Figure 2.13 - Absolute bead counts
- Figure 2.14 - Regulatory T cells (Tregs)
- Figure 2.15 - CD4+ T stages
- Figure 2.16 - CD8+ T stages
- Figure 2.17 – B stages
- Figure 2.18 – B stages - CD10 negative
- Figure 2.19 - Recent thymic emigrants – CD4+
- Figure 2.20 - Recent thymic emigrants – CD8+

Figure 2.10 - Database – Main page

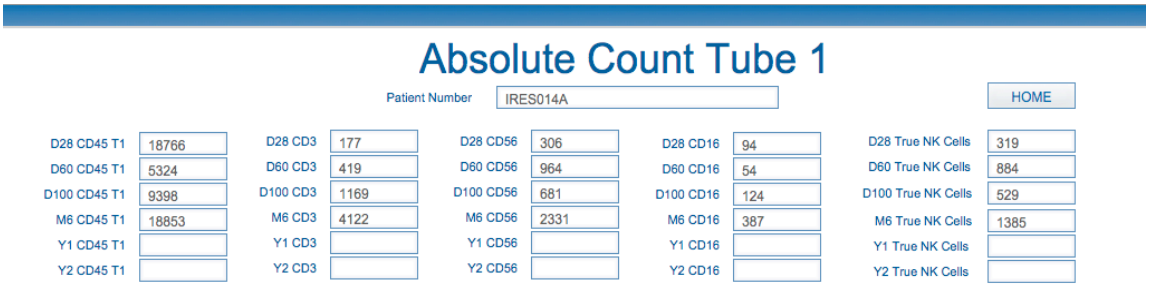


Figure 2.11 Database - Absolute counts 1

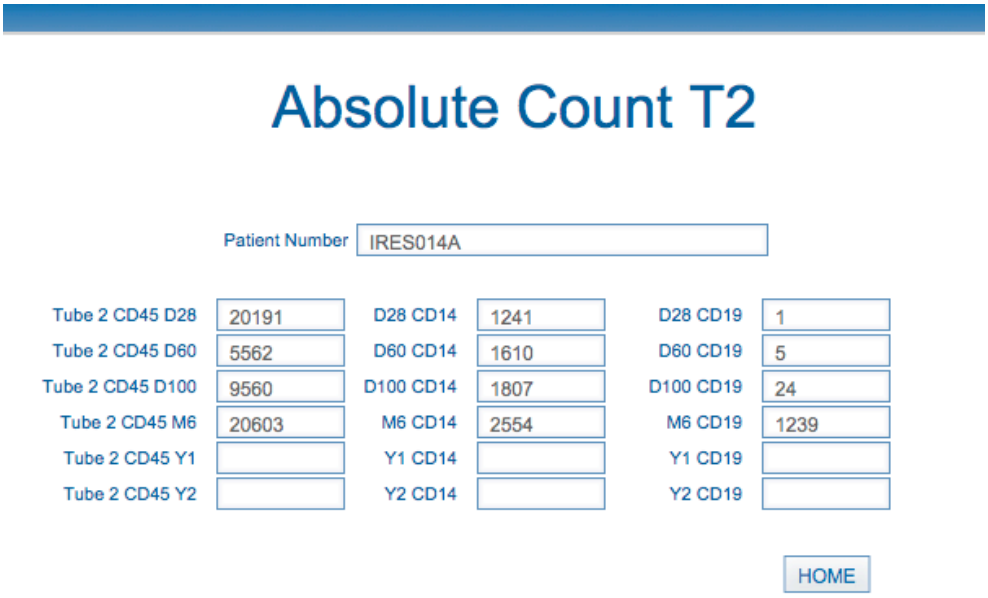


Figure 2.12 Absolute counts 2

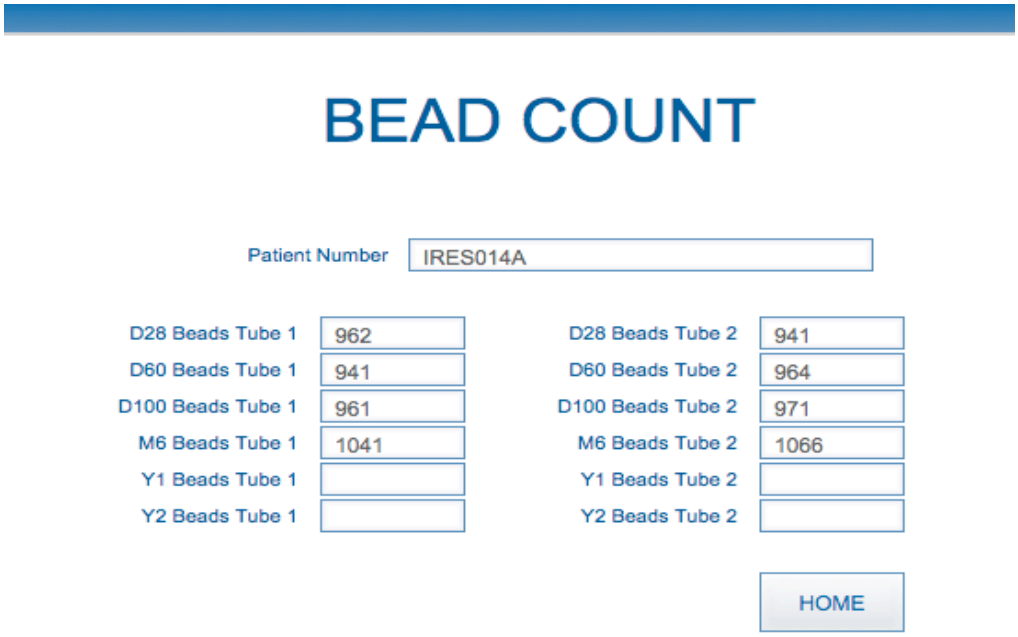


Figure 2.13 Database - Bead Count

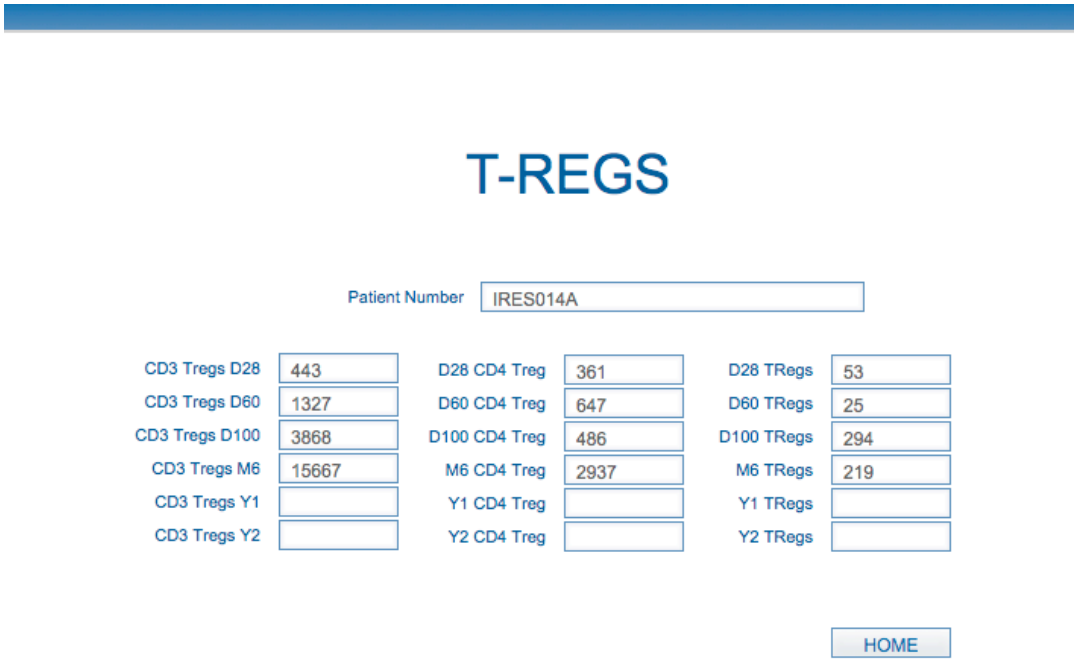


Figure 2.14 Database - Tregs

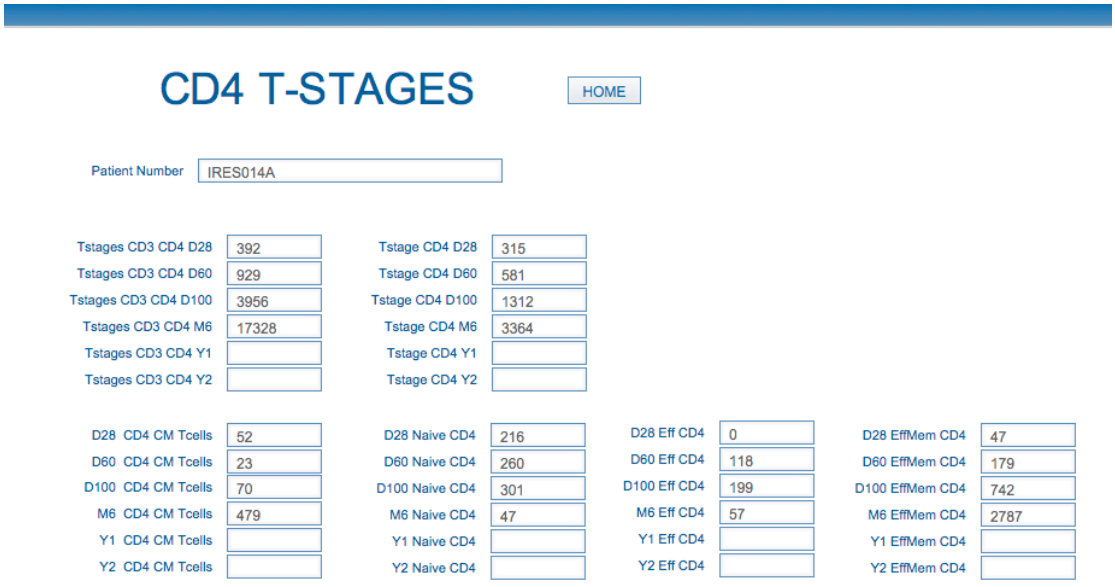


Figure 2.15 Database - CD4+ T cell stages

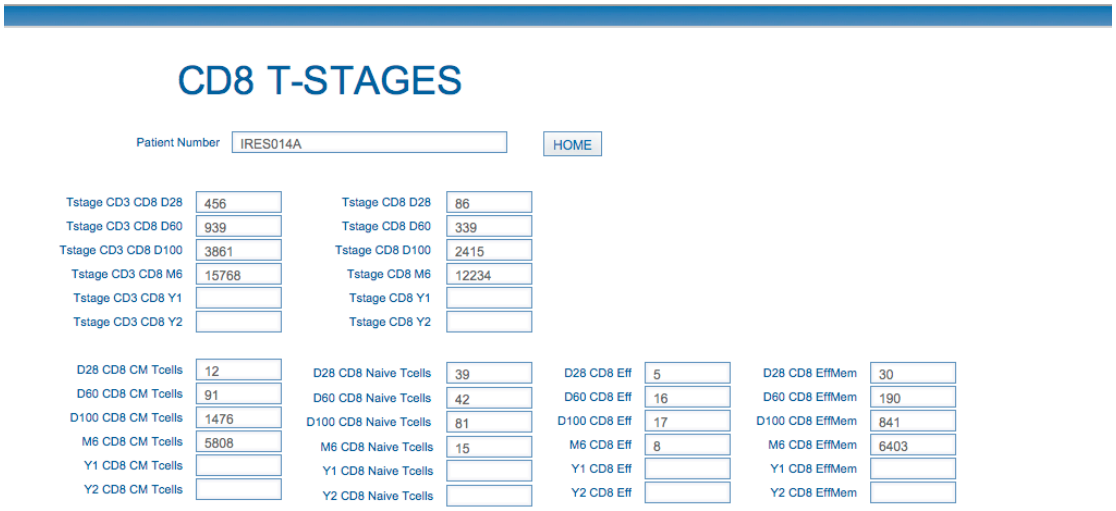


Figure 2.16 Database - CD8 T stages

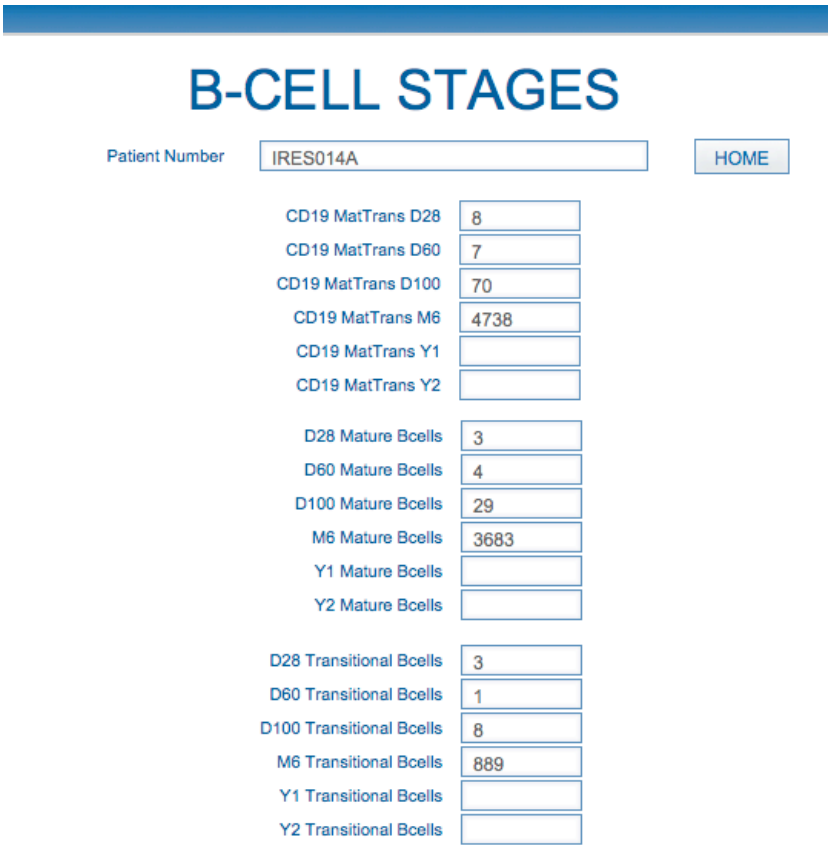


Figure 2.17 Database - B cell stages

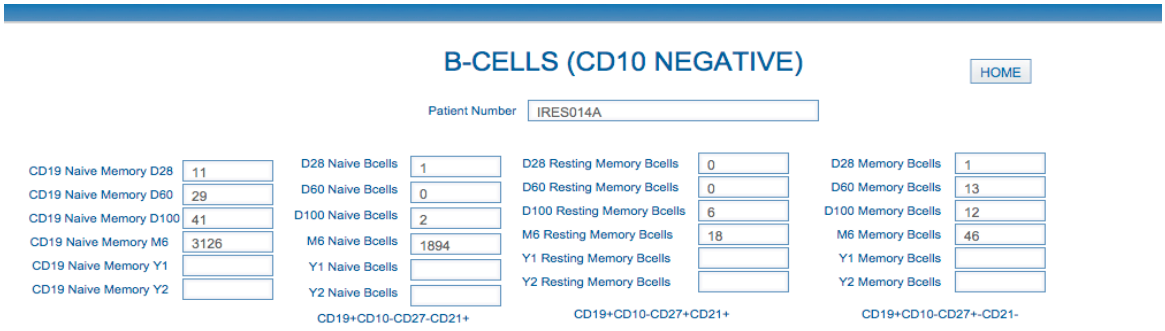


Figure 2.18 Database - B cell stages (CD10-)

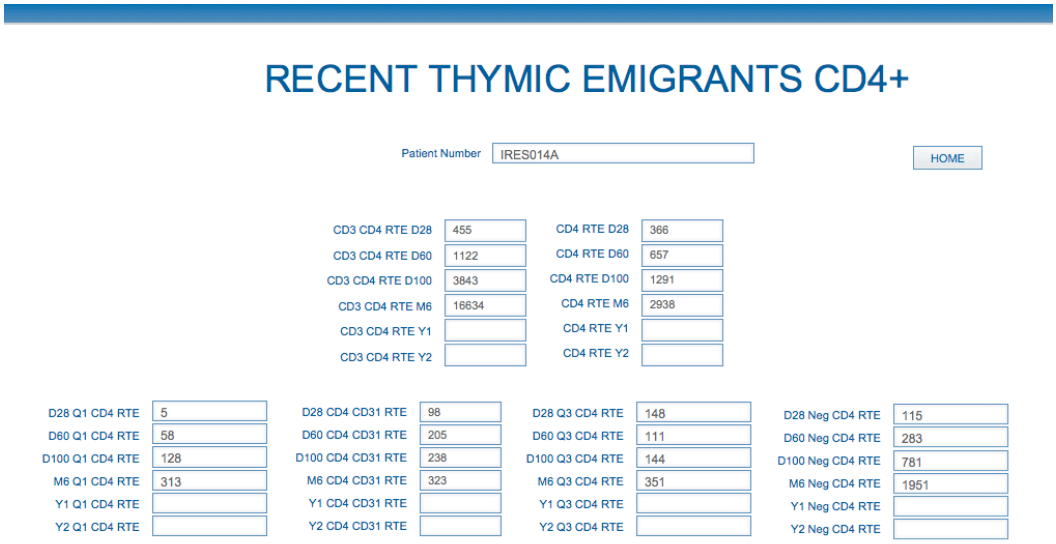


Figure 2.19 Database - CD4+ T cells - Recent thymic emigrants

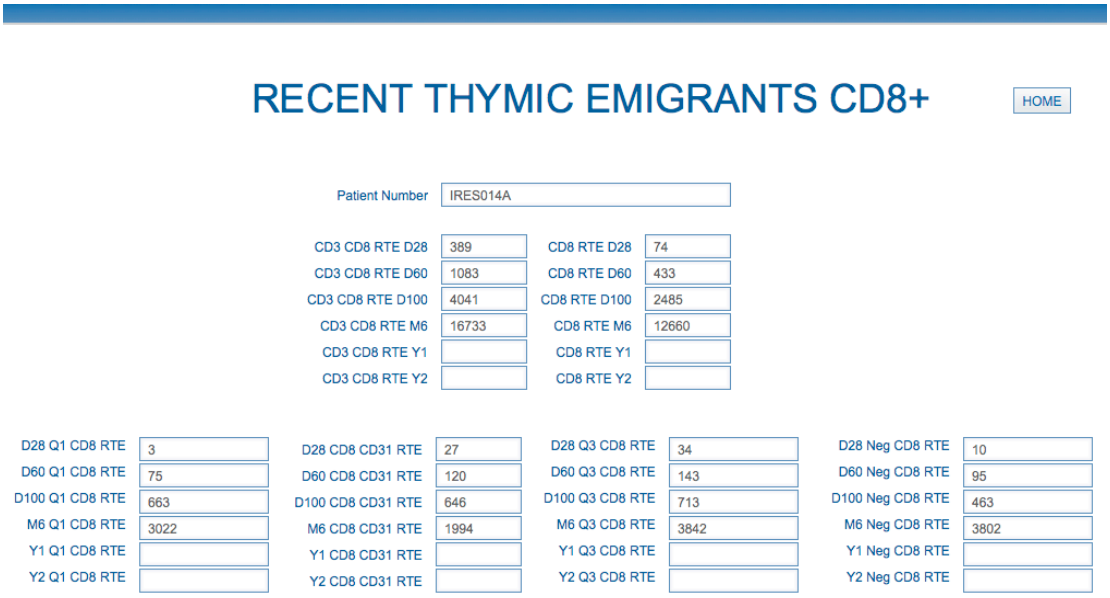


Figure 2.20 Database - CD8+ T cells - Recent thymic emigrants

2.6 TruCount calculator

To accurately quantify the absolute count of cells within patient samples a TruCount calculator was devised using FileMaker Pro. To quantify the number of cells, the relevant volumes of antibodies and respective chemical volumes are input into the system. The calculator performed various calculations and the final value given is a cells/microliter reading of the desired cell subset. This can be seen in Figure 2.21.

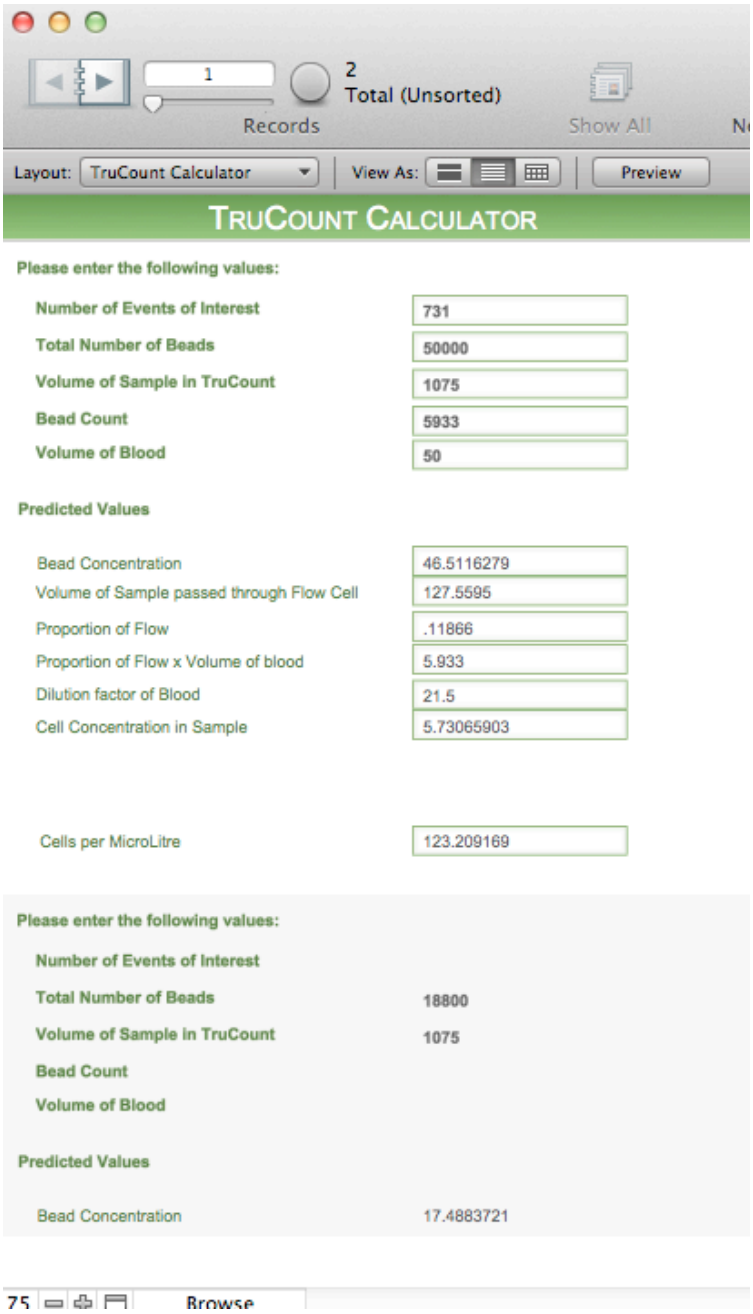


Figure 2.21 TruCount Calculator produced on Filemaker Pro

2.7 Methods for molecular studies

2.7.1 Genomic DNA extraction

DNA was extracted from healthy adult PBMCs and patients PBMCs, as per the QIAamp DNA blood Mini Kit protocol.

2.7.2 Quantification of nucleic acid

DNA concentrations were quantified using the Nanodrop ND-1000 spectrophotometer (Labteach, Lews, UK). This was undertaken as per the manufacturer's recommendation. The purity of the sample was assessed by a ratio of absorbance OD_{260nm}:$OD_{280nm.}$ A ratio between 1.6 and 1.8 would be considered satisfactory. Samples were then stored at 4^{o}C for short-term storage or -20^{o}C for long-term storage. The DNA concentration used for this study was 100 ng/mL.

2.7.3 Plasmid extraction

The TOPO 2.1 plasmid (Figure 2.22) was donated by Dr A Sottini (CREA, Diagnosis Department, Spedali Civili di Brescia), which contained the triple insert for TREC, KREC and TRAC. The plasmid was bound to Whatmann paper and this was extracted by placing the Whatmann paper into TE buffer within an Eppendorf tube. Thereafter the Eppendorf tube was placed on a roller for three to six hours. Once diluted, 2 µL of diluted plasmid was used for transformation into E.Coli.

2.7.3.1 Plasmid map of pCR 2. 1 TOPO

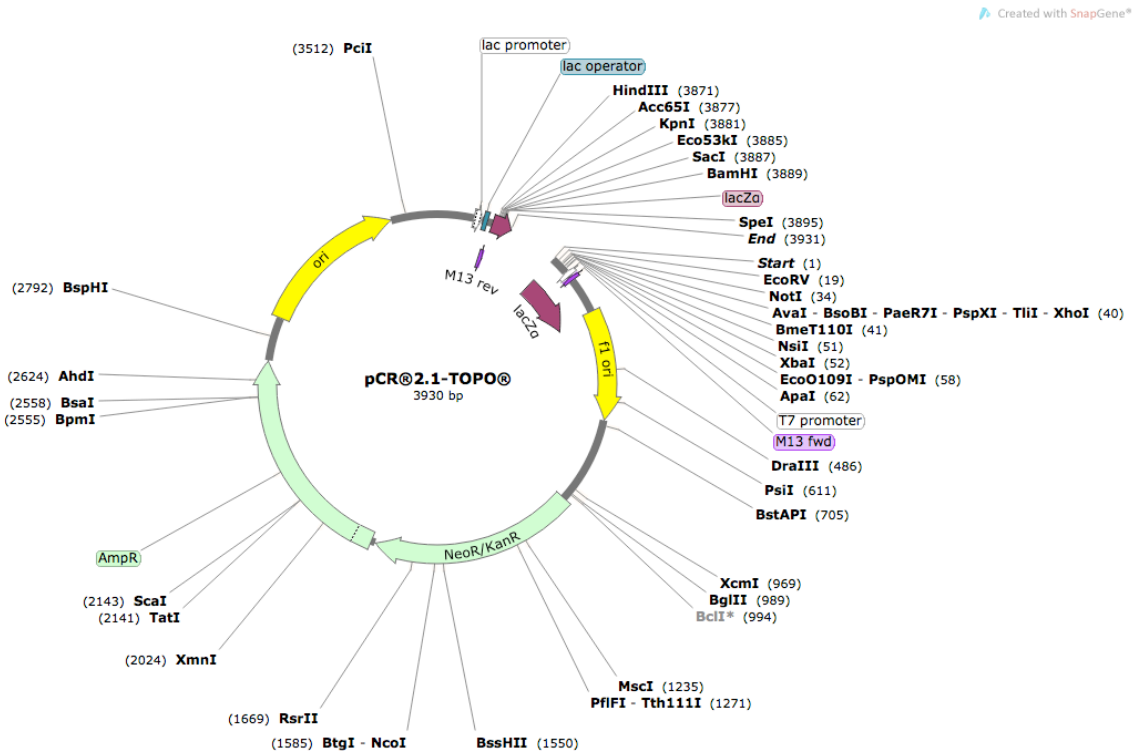


Figure 2.22 Plasmid map of pCR-2.1 TOPO. This has multiple cloning sites and was used for expression of TREC, KREC and TRAC. The insertion of TREC, KREC and TRAC are within the T-A acceptor site of pCR2.1-TOPO Vector. Plasmid utilised for T-cell receptor excision (TREC) circle quantification and Kappa deleting excision circle (KREC) quantification. The arrows indicate the direction of transcription. Source of Image: https://www.addgene.org/PR/091911/

2.7.4 Transformation into competent cells

Oneshot TOP10 competent cells (Invitrogen, Life Technologies, Carlsbald, California) were used to transform the triple-insert plasmid. To perform the transformation chemically the rapid chemical transformation procedure was undertaken as per manufacturer's instructions.

2.7.5 Culture for plasmid extraction

Transformed colonies were selected and placed into falcon tubes containing Laura Broth (LB) with Kanamycin. The falcon tubes were then placed into a shaking incubator at 2000 rpm at 37^{o}C, overnight.

2.7.6 Glycerol stocks of plasmids

800 µL of transformed colony culture was added to two to four drops of autoclaved glycerol within a cryovial. The cryovial was shaken for one minute and then stored at -20^{o}C until further plasmid stocks were required.

2.7.7 Plasmid preparation

Plasmid extraction and preparation was performed using the PureYield Plasmid Miniprep Kit as per the manufacturer's instructions.

2.7.8 Plasmid restriction digestion

Two restriction digests were performed to ensure that the plasmid was linearised prior to sequencing for the TREC, KREC and TRAC genes. Plasmid DNA was linearised using Xho I or EcoRI restriction enzymes.

2.7.9 Gel electrophoresis

100 mL of 1X TBE buffer and 1.5g of agarose were used to produce a gel, which were mixed and heated in a microwave. The solution was then cooled and 5 µL of 1000X Ethidium Bromide (0.5 mg/ml) was then added. The gel was cast and the linearised gene sequences were then run on the gel for 45 minutes at 150V.

2.7.10 Cycle sequencing reaction

Cycle sequencing was undertaken with the TREC/KREC or TRAC genes within the plasmid. Table 2.13 defines the master mix reaction used for the cycle sequencing reaction.

Component	Volume
Ready Reaction Mix	1 µL
5X Buffer	0.5 µL
Primer	1 µL
Distilled Water	2.5 µL
Plasmid DNA	5 µL

Table 2.13 Mastermix for the cycle sequencing reaction

2.7.11 Precipitation of cycle sequenced products and ABI loading

Once the cycle sequencing was performed the products were precipitated before they could be sequenced via the ABI sequencer. 2.5 µL of EDTA (125 mM) and 30 µL of 100% ethanol were added to each well before being vortex mixed. The sequencing plate was incubated at room temperature for 15 minutes followed by a 3200 rpm centrifugation for 30 minutes. The plate was then unsealed and inverted onto tissue and centrifuged at 900 rpm for three seconds. 30 µL of 70% ethanol was then added to each well. The plate was gently flicked to mix the contents within each well and centrifuged at 900 rpm for 3 seconds. The plate was air dried for 5 minutes and all the wells were rehydrated with 10 µL of HiDi solution (Applied Biosystems). The plate was then vortexed before a septa seal was placed over the wells. The plate was placed into the sequencing cassette and loaded into the ABI sequencer.

2.7.12 Microelectrophoresis of sequences

All sequencing data was generated via capillary electrophoresis using an ABI GA-3730xl genetic analyser. The sequencing platform is based on 96 well glass coated capillaries, 50 cm, and is filled with a polymer (POP-7). The runs on the

sequencer are performed at 60^{o}C with an injection time of 15 seconds. The injection has a voltage of 1.5kV and a running voltage of 8.5kV. All settings were kept as recommended by the manufacturer.

2.7.13 Analysis of the sequencing data

The data generated post-capillary electrophoresis was transferred to a computer and analysed using Sequencing Analysis 5.2 or Seq Scape2.5.0 Software (PE Applied Biosystem, Foster City, CA, USA). The data was then inverted and inserted into Factura (PE Applied Biosystem, Foster City, CA, USA). The sequence was then imported into Sequence Navigator (PE Applied Biosystem, Foster City, CA, USA) to identify the quality of the sequence generated. The average base pair read for TREC, KREC and TRAC was 600 bp. The sequences were overlapped and aligned. A consensus sequence was produced and computed, thereafter being exported into a text file and pasted into a word document to be aligned with the primer sequences. BLAST searches were then performed to confirm 100% sequence identity within the plasmid samples.

2.7.14 Primer/probe gene sequences for TREC, KREC and TRAC quantification via RT-PCR

All primers used in TRAC, TREC and KREC quantification are listed in Table 2.14.

Gene		Sequence 5'-3'
TREC	forward	CAC ATC CCT TTC AAC CAT GCT
	reverse	TGC AGG TGC CTA TGC ATC A
	probe	FAM-ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT-TAMRA
KREC	forward	TCC CTT AGT GGC ATT ATT TGT ATC ACT
	reverse	AGG AGC CAG CTC TTA CCC TAG AGT
	probe	HEX-TCT GCA CGG GCA GCA GGT TGG-TAMRA
TRAC	forward	TGG CCT AAC CCT GAT CCT CTT
	reverse	GGA TTT AGA GTC TCT CAG CTG GTA CAC
	probe	FAM-TCC CAC AGA TAT CCA GAA CCC TGA CCC-TAMRA

Table 2.14 Primer and Probe sequences for TREC, KREC and TRAC genes

2.7.15 Cell isolation procedures for T-cell Receptor Excision Circles (TREC) / Kappa chain Receptor Excision Circles (KREC)

For the cells that were subjected to TREC and KREC quantification methods, three isolation methods were used to distinguish whether valid results can be achieved through differential cell isolation procedures, listed below.

- Monoclonal antibody isolation via cell sorting
- Magnetic bead isolation
- Ficoll separation

2.7.16 Monoclonal antibody isolation

Cells were isolated using ficoll methods as described earlier. 10 x 10^6 isolated cells were stained with monoclonal antibodies against anti-FITC for CD4 / CD8 (Miltenyi, Germany Cat No: 130-048-071), anti-APC for CD19 (Miltenyi, Germany Cat No: 130-090-855). Cells were stained at 4^{o}C for 20 minutes and then washed at 1300 rpm for 5 minutes twice. Cells were re-suspended in 500 µL of FACS buffer at each wash step. The cells were strained using a BD Falcon 70 µm cell strainer for 50 mL falcon tubes, prior to being sorted on the FACS Aria (BD, UK) within the Immunology department of the Royal Free Hospital. The respective cell subsets were then frozen for DNA extraction.

2.7.17 Magnetic bead isolation

Cells were isolated using ficoll methods described earlier. To further isolate certain subsets a vial of 10 x 10^6 isolated frozen CB cells were thawed and stained with monoclonal antibodies against CD4 (FITC), CD8 (FITC) and CD19 (APC). Cells were then placed at 4^{o}C for 20 minutes. The cells were then washed with 1 mL of MACS buffer and centrifuged at 1300 rpm for 5 minutes. This step was repeated twice. The cells were then re-suspended in 100 µL of MACS buffer. Cells were then subjected to staining with anti-FITC microbeads (130-048-70) (Miltenyi, Germany) or anti-APC conjugated microbeads. 10 µL of the conjugated beads were added to the cells, which were then incubated for 10

minutes in the dark at 4°C-8°C. The cells were then washed in 2 mL of MACS buffer and centrifuged at 1800 rpm for 10 minutes. The supernatant was then discarded and the cells are re-suspended in 500 µL of MACS buffer.
Magnetic separation was then undertaken using a MACs column and MACS separator (Miltenyi, Germany). The CD4, CD8 and CD19 cell subsets were extracted using LS columns (130-042-401) (Miltenyi, Germany), which were used for positive selection of the desired cells. The cells were isolated as per the manufactures instructions and tested for purity and frozen for DNA extraction.

2.7.18 Mononuclear isolation

This was performed as described in section 2.3.2.

2.7.19 Standard curve preparation for TREC/KREC and TRAC real time - PCR

A plasmid standard curve is used in RT-PCR to efficiently quantify and qualify the gene of interest. The triple inserted plasmid serves as a control to quantify TREC, KREC and TRAC copy numbers. Firstly, the mass of the plasmid copy numbers was calculated. This took into account that the plasmid size was 4,846bp in length. The average mass per base pair was 1.096×10^{21} g/bp.

Therefore, the mass of plasmid copy number is the following:

$(4{,}846 \text{ bp}) \times (1.096 \times 10^{-21} \text{ g/bp}) = 5.311 \times 10^{-18}\text{g}$

Using this mass the following table, Table 2.15, shows the mass per copy numbers required at each concentration.

Copy Number		Mass of Plasmid DNA (g)
1×10^{6}	X 5.311×10^{-18} g	**5.311×10^{-12}**
1×10^{5}		**5.311×10^{-13}**
1×10^{4}		**5.311×10^{-14}**
1×10^{3}		**5.311×10^{-15}**
1×10^{2}		**5.311×10^{-16}**

Table 2.15 Mass of plasmid required for each standard curve dilution point

Using the mass of plasmid concentrations (Table 2.15), the concentrations of plasmid DNA were calculated (Table 2.6) by dividing the masses by the volume to be pipetted into each reaction as shown below in Table 2.17.

Mass of Plasmid DNA (g)		Final concentration of plasmid DNA (g/µL)
5.311×10^{-12}	÷5	**1.062×10^{-12}**
5.311×10^{-13}		**1.062×10^{-13}**
5.311×10^{-14}		**1.062×10^{-14}**
5.311×10^{-15}		**1.062×10^{-15}**
5.311×10^{-16}		**1.062×10^{-16}**

Table 2.16 Concentration of plasmid DNA

An aliquot of plasmid was thawed and linearised using restriction enzyme Xho I. The concentration was determined via spectrophotometric estimation at 260nm/280nm. A working stock of $1x10^{-10}$ g/µL was prepared. The following dilutions were made so that as shown in Table 2.17.

Initial concentration (g/µL)	Plasmid DNA (µL)	Diluent Volume (µL)	Final Volume (µL)	Final Concentration (g/µL)	Final Copy Number of Plasmid DNA / 5 µL
$1x10^{-7}$	5	45	50	$1x10^{-8}$	-
$1x10^{-8}$	5	495	500	$1x10^{-10}$	-
$1x10^{-10}$	5	465	470	$1.062x10^{-12}$	$1x10^{6}$
$1.062x10^{-12}$	5	450	500	$1.062x10^{-12}$	$1x10^{5}$
$1.062x10^{-13}$	5	450	500	$1.062x10^{-12}$	$1x10^{4}$
$1.062x10^{-14}$	5	450	500	$1.062x10^{-12}$	$1x10^{3}$
$1.062x10^{-15}$	5	450	500	$1.062x10^{-12}$	$1x10^{2}$

Table 2.17 Working concentrations of plasmids

The highest dilutions of 10 copies/ 5 µL were prepared before the assay as they contain a small quantity of plasmid DNA and are not stable enough to be stored for future use.

The plate layout used to quantify TREC, KREC and TRAC genes in patient and healthy control samples is shown in Figure 2.23

	TREC+KREC		TRAC		TREC+KREC		TRAC		TREC+KREC		TRAC	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	10^6 copies	10^6 copies	10^6 copies	10^6 copies	1	1	1	1	9	9	9	9
B	10^5 copies	10^5 copies	10^5 copies	10^5 copies	2	2	2	2	10	10	10	10
C	10^4 copies	10^4 copies	10^4 copies	10^4 copies	3	3	3	3	11	11	11	11
D	10^3 copies	10^3 copies	10^3 copies	10^3 copies	4	4	4	4	12	12	12	12
E	10^2 copies	10^2 copies	10^2 copies	10^2 copies	5	5	5	5	13	13	13	13
F	10 copies	10 copies	10 copies	10 copies	6	6	6	6	14	14	14	14
G	+ve control	+ve control	+ve control	+ve control	7	7	7	7	15	15	15	15
H	-ve control	-ve control	-ve control	-ve control	8	8	8	8	16	16	16	16

Figure 2.23 RT-PCR plate layout for TREC/KREC and TRAC. The respective plate layout was used to quantify the copy numbers of TRAC, TREC and KRECs in healthy controls and patients.

The following master mix solutions were performed and added to the respective wells as shown in Table 2.18 below.

TREC/KREC		TRAC	
H_20	2.0 µL	H_20	4.75 µL
KRECs forward	1.125 µL	KRECs forward	2.0 µL
KRECs reverse	1.125 µL	KRECs reverse	2.0 µL
KRECs probe	0.5 µL	KRECs probe	2.0 µL
TRECs forward	1.125 µL	2x TaqMan Universal Master Mix	2.0 µL
TRECs reverse	1.125 µL		
TRECs Probe	0.5 µL		
2x TaqMan Universal Master Mix	12.5 µL		

Table 2.18 Mastermix solutions for TREC/KREC and TRAC

20 µL of the respective master mixes and 5 µL of genomic DNA were added to the respective wells.

The real-time PCR plate was then run using the Bio-Rad CFX96 Real-Time System. The protocol set for the TREC run was the following:

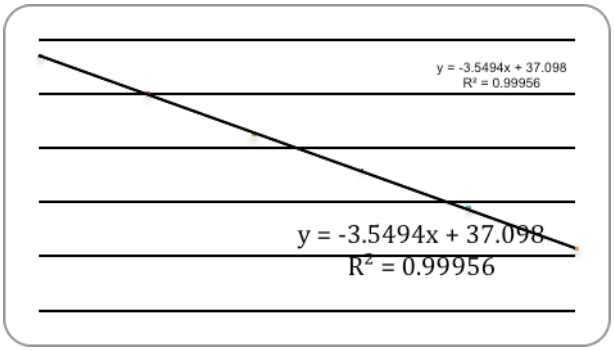
- 50^{o}C for 2 minutes
- Initial heating at 95^{o}C for 10 minutes
- Forty-five denaturation cycles at 95^{o}C for 15 seconds
- Combined primer/probe annealing and elongation at 60^{o}C for 1 minute

2.7.20 Real time PCR standard curve calculations

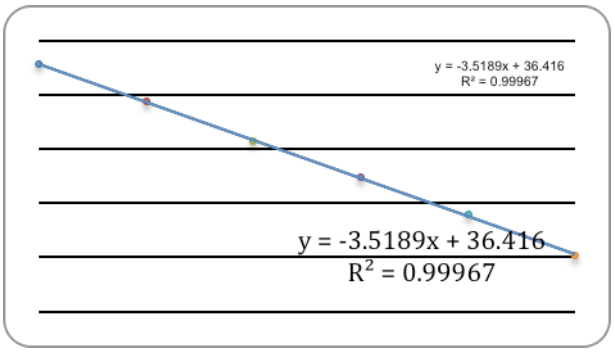
The data from the Bio-Rad CFX96 Real-Time System was then exported and before quantification of TREC, KREC and TRAC could be undertaken a standard curve for TREC, KREC and TRAC was generated. These were undertaken to verify the ideal exponential amplification rate, which would correspond to the RT-PCR efficiency.

The following graphs represent standard curves generated to identify efficiency for a real-time PCR reaction.

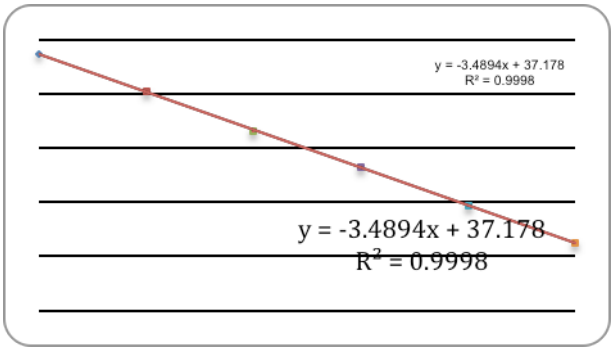
TRAC



TREC



KREC



2.7.21 PCR efficiency calculation

PCR efficiency was calculated for each reaction using the plasmid lines of best fit for TRAC, TREC and KREC. This was to ensure that during the logarithmic phase of the PCR reaction the PCR product of interest is doubling with each cycle. To calculate the PCR efficiency, for each plasmid standard curve a line of best-fit equation was generated using Microsoft Excel. The equation was usually in the format of y = - mx+c. The –m value was input into the following equation to calculate the efficiency of the PCR:

$$\textbf{Efficiency of PCR} = (10^{(-1/-m)}-1) \times 100$$

Further calculations of copy numbers of genes were only calculated if the PCR efficiency was above 90%.

2.7.22 Calculations and quantification of TRAC, TREC and KREC

To quantify the copy number of the TRAC, TREC and KREC genes the CT values were input into the plasmid standard curve equation. The calculation was performed as follows:

1. Using the duplicate values for each patient sample an average value is calculated
2. Using values from the slope gradient equations y = -mx+ c the following equation is constructed
3. ((Average patient CT value – c)/ -m)) x 10^7
4. The value calculated is the mean quantity TREC/KRECs within the patient samples
5. The following formula should be used to calculate the copy number of TREC/KREC

((Mean quantity of TREC or KREC / Mean quantity of TRAC/ 2)) x10^7

The mean quantity of TRAC was divided by two as each cell had two gene copies, one for each chromosome.

2.8 Methods for functional studies

2.8.1 Cell surface staining protocol for immunophenotyping for functional cell analysis

Isolated healthy control and patient PBMCs were thawed as described in 2.3.6. The cells were subjected to cell surface staining as described in 2.3.8. The cells were acquired on a flow cytometer as previously described in section 2.3.10.

2.8.2 Intracellular staining for IFN- γ

Healthy control and patient PBMCs were thawed, as described in 2.3.6. The cells were surfaced stained in 50 µL of an antibody cocktail consisting of CD56, CD16, CD3, CD14, HLA-DR and CD69. The cells were centrifuged at 1500 rpm at room temperature and the supernatant was discarded. The cells were re-suspended in 100 µL of BD Cytofix/CytoPerm solution and incubated for 20 min in the dark at 4-8^{o}C. The cells were centrifuged at 1500 rpm for three minutes and washed in 200 µL of Perm/Wash Buffer. The supernatant was discarded and the cells were resuspended in 100 µL of IFN-γ antibody cocktail, which was freshly prepared at a 1/100 dilution within Perm/Wash buffer. The cells were incubated at 4^{o}C for 30 minutes and washed twice in Perm/Wash buffer prior to centrifugation at 1500 rpm for three minutes. The supernatant was discarded and the cells were re-suspended in 400 µL of FACS buffer. The cells were transferred to Falcon round-bottom tubes for acquisition on a flow cytometer.

2.8.3 Intra-nuclear staining for Foxp3

Healthy and patient PBMCs were thawed as described in 2.3.6. The cells were surface stained in 50 µL of CD4, CD3, CD25 and CD127 for 15 minutes at room temperature. The cells were collected via centrifugation at 1500 rpm for four minutes at 4^{o}C and the supernatant was discarded. The cells were re-suspended in 100 µL of FoxP3 fixation/permeabilization buffer and incubated for 30 mins at 2-8^{o}C in the refrigerator, in the dark. The cells were collected via centrifugation at 1500 rpm, for 4 mins, at 4^{o}C and the supernatant was discarded. The cells were washed in 80 µL FoxP3 permeabilization buffer and

centrifuged at 1500 rpm for 4 minutes at 4^{o}C, which was repeated twice. The cells were resuspended in 50 µL of FoxP3 antibody and incubated for 30 mins at 2-8^{o}C in the refrigerator, in the dark. The cells were collected via centrifugation at 1500 rpm for four mins, at 4^{o}C and the supernatant was discarded. The wash step was then repeated twice and the cells were then re-suspended in 100 µL FACS buffer before being transferred to 5 mL round-bottomed tubes and stored at 4^{o}C prior to acquisition on a flow cytometer.

2.8.4 Immunophenotyping to identify functional cellular subsets

Thawed healthy control and patient PBMCs were used for Immunophenotyping to understand the function of immune cell subsets in CB transplanted patients. Immunophenotyping via flow cytometry was performed to distinguish the immune cells and respective functional markers:

- Alpha-Beta and Gamma-delta T-cells
- T cell activation markers
- NK cell activation markers
- NK cell activation and inhibitory markers
- NK cell IFN-gamma expression
- Regulatory T cells

Respectively the phenotypes for these populations were the following:

1. Alpha Beta and Gamma Delta T-cells
   a. $CD3^{+}$ $CD4^{+}$ TCR αβ
   b. $CD3^{+}$ $CD4^{+}$ TCR γδ
   c. $CD3^{+}$ $CD8^{+}$ TCR αβ
   d. $CD3^{+}$ $CD8^{+}$ TCR γδ
   e. $CD3^{+}$ $CD4^{-}$ CD8- TCR αβ and TCR γδ

2. T cell activation markers – CD4+ and CD8+ T cells
   a. CD4+ T cells
      i. $CD3^{+}$ $CD4^{+}$ $HLA\text{-}DR^{+}$
      ii. $CD3^{+}$ $CD4^{+}$ $CD69^{+}$

iii. $CD3^+ CD4^+ CD25^+$

b. CD8+ T cells

i. $CD3^+ CD8^+ HLA\text{-}DR^+$

ii. $CD3^+ CD8^+ CD69^+$

iii. $CD3^+ CD8^+ CD25^+$

3. NK-cell/ T cell and NK-T cell activation markers

a. $CD3^- CD56^{bright} CD16^{negative}$

i. HLA-DR+

ii. CD69+

b. $CD3^- CD56^{dim} CD16^{positive}$

i. HLA-DR+

ii. CD69+

c. $CD3^- CD56^{dim} CD16^{negative}$

i. HLA-DR+

ii. CD69+

4. NK cell IFN-gamma expression

a. $CD3^- CD56^{bright} CD16^{negative}$ IFN-γ^+

b. $CD3^- CD56^{dim} CD16^{positive}$ IFN-γ^+

c. $CD3^- CD56^{dim} CD16^{positive}$ IFN-γ^+

5. Activating and inhibitory markers of NK cells

a. $CD3^- CD56^{bright} CD16^{negative}$

i. NKG2A

ii. NKG2C

iii. CD57

b. $CD3^- CD56^{dim} CD16^{positive}$

i. NKG2A

ii. NKG2C

iii. CD57

c. $CD3^- CD56^{dim} CD16^{negative}$

i. NKG2A

ii. NKG2C

iii. CD57

6. Regulatory T cells – $CD3^+CD4^+CD25^+CD127^{low}$ $Foxp3^+$

2.8.4.1 Gating strategies for functional immunophenotyping analysis

The following gating strategy (Figure 2.24) was used to analyse and identify Alpha Beta and Gamma Delta T cells in thawed healthy control and patient PBMC samples.

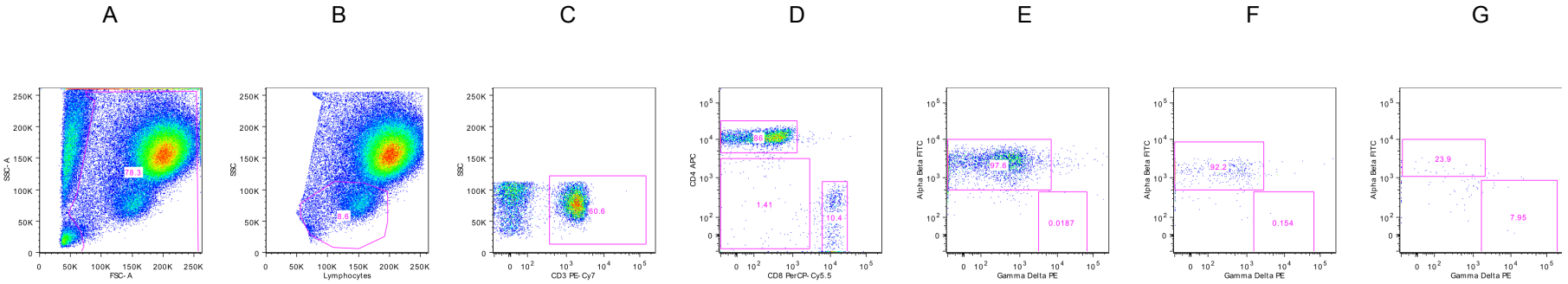


Figure 2.24 Gating strategy for Alpha Beta and Gamma Delta T cells. Thawed healthy control and patient PBMCs were stained with monoclonal antibodies for surface expression of CD3, CD4, CD8, TCR αβ and TCR γδ. (A) Gating of mononuclear cells, minus the debris, (B) Gating of lymphocytes, (C) Gating of $CD3^+$ T cells from the lymphocyte gate, (D) Gating of CD4+, CD8+ T cells and $CD4^-CD8^-$ T cells (E) Gating of TCR αβ and TCR γδ within the $CD4^+$ T cell population, (F) Gating of TCR αβ and TCR γδ within the $CD8^+$ T cell population (G) Gating of TCR αβ and TCR γδ within the $CD4^-CD8^-$ T cells population.

The following gating strategy shown in Figure 2.25 was used to analyse and identify activation markers, CD25, CD69 and HLA-DR on CD4 and CD8 T cells in healthy control and patient PBMC samples.

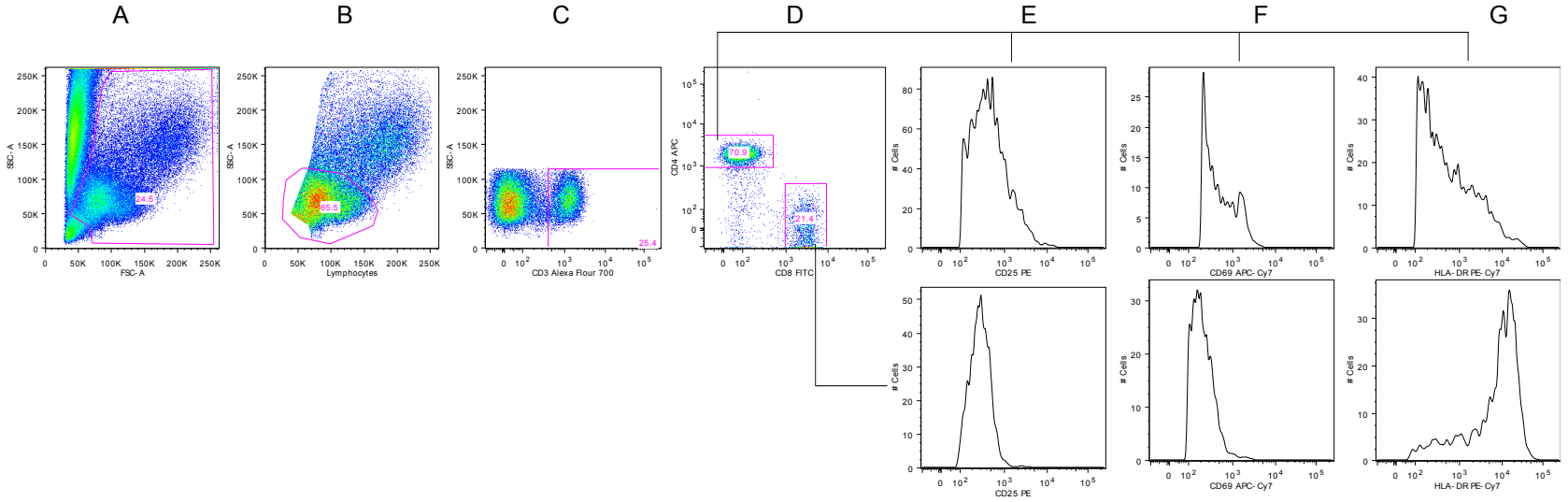


Figure 2.25 Gating strategy for T cell activation markers. Thawed healthy control and patient PBMCs were stained with monoclonal antibodies for surface expression of CD3, CD4, CD8, CD25, CD69 and HLA-DR. (A) Gating of mononuclear cells, minus the debris, (B) Gating of lymphocytes, (C) Gating of $CD3^{+}$ T cells from the lymphocyte gate, (D) Gating of CD4+ and CD8+ T cells, (E) Gating of CD25 within CD4+ and CD8+ T cells, (F) Gating of CD69 within CD4+ and CD8+ T cells, (G) Gating of HLA-DR within CD4+ and CD8+ T cells.

The following gating strategy shown in Figure 2.26 was used to analyse and identify activation markers CD69 and HLA-DR on NK cell subsets in healthy control and patient PBMCs. Both CD69 and HLA-DR are analysed on $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cell subsets.

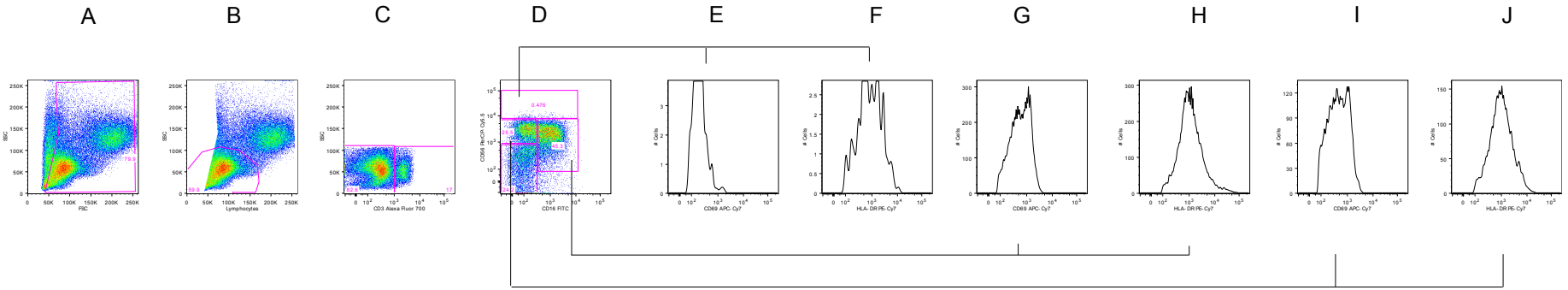


Figure 2.26 Gating strategy for NK cell activation markers. Thawed healthy control and patient PBMCs were stained with monoclonal antibodies for surface expression of CD3, CD16, CD56, CD69 and HLA-DR. (A) Gating of mononuclear cells, minus the debris, (B) Gating of lymphocytes, (C) Gating of $CD3^-$ from the lymphocyte gate, (D) Gating of $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cells, (E) Gating of CD69 within $CD56^{bright}$ $CD16^{dim}$ NK cells, (F) Gating of HLA-DR within $CD56^{bright}$ $CD16^{dim}$ NK cells, (G) Gating of CD69 within $CD56^{dim}$ $CD16^{positive}$ NK cells, (H) Gating of HLA-DR $CD56^{dim}$ $CD16^{positive}$ NK cells, (I) Gating of CD69 within $CD56^{dim}$ $CD16^{negative}$ NK cells, (J) Gating of HLA-DR $CD56^{dim}$ $CD16^{negative}$ NK cells.

The following gating strategy shown in Figure 2.27 was used to analyse and identify the intracellular expression of IFN-ɣ within $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cell subsets of healthy controls and patient PBMCs.

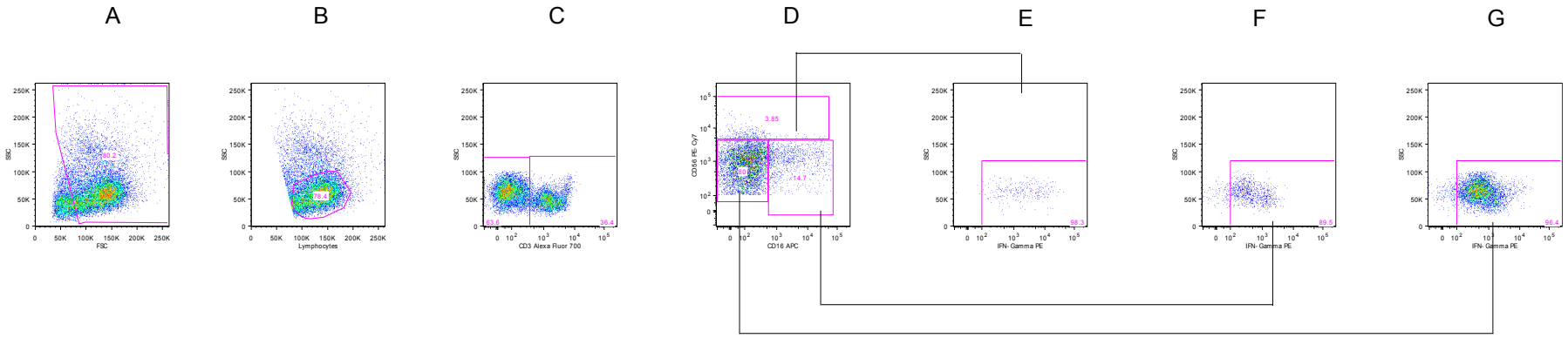


Figure 2.27 Gating strategy for intracellular IFN-ɣ within NK cells. Thawed healthy control and patient PBMCs were stained with monoclonal antibodies for surface expression of CD3, CD16, CD56 and IFN-ɣ (A) Gating of mononuclear cells, minus the debris, (B) Gating of lymphocytes, (C) Gating of $CD3^{-}$ from the lymphocyte gate, (D) Gating of $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cells, (E) Gating of IFN-ɣ within $CD56^{bright}$ $CD16^{dim}$ NK cells, (F) Gating of IFN-ɣ within the $CD56^{dim}$ $CD16^{positive}$ NK cells (G) Gating of IFN-ɣ within $CD56^{dim}$ $CD16^{negative}$ NK cells.

The following gating strategy shown in Figure 2.28 was used to analyse and identify the expression of inhibitory and activation markers, NKG2C, NKG2A and a functional maturation marker, CD57, within $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cell subsets within healthy control and patient PBMCs.

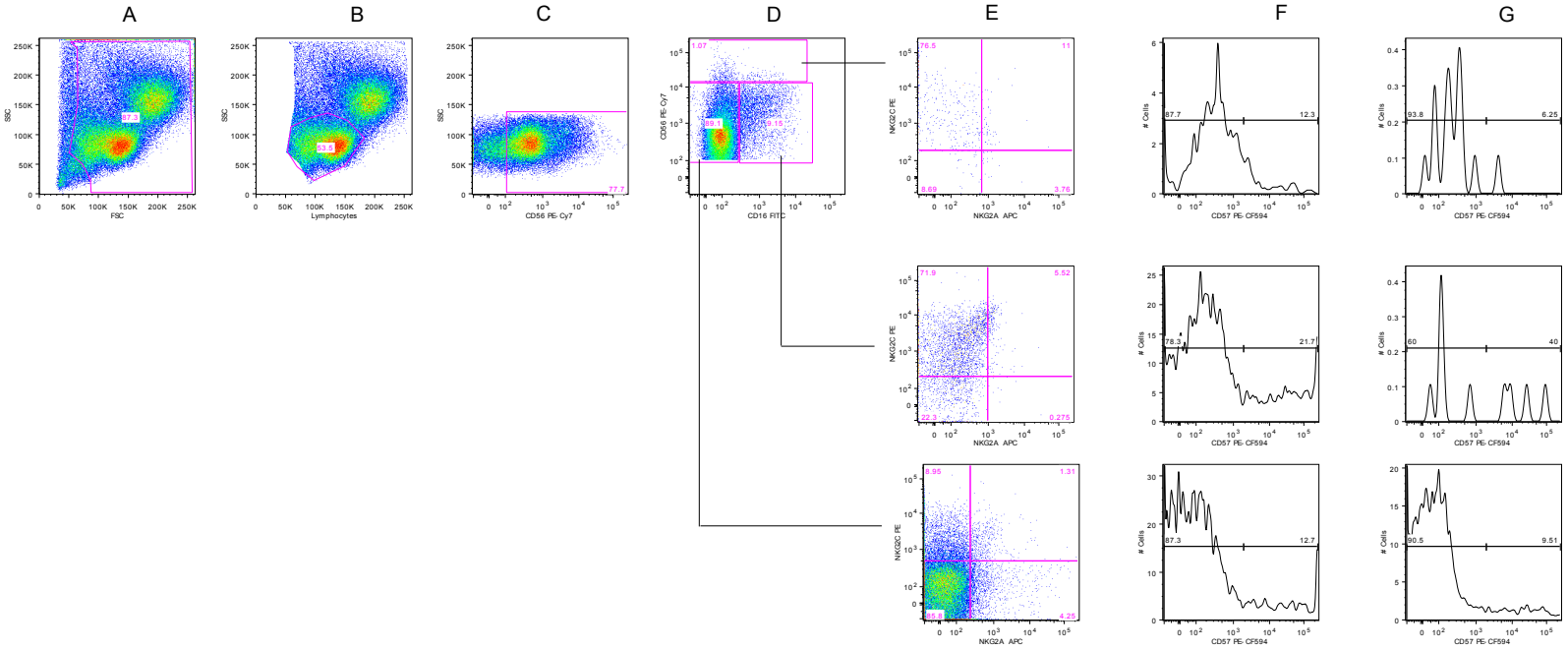


Figure 2.28 Gating strategy to identify the expression of inhibitory and activation markers within NK cells. Thawed healthy control and patient PBMCs were stained with monoclonal antibodies for surface expression CD16, CD56, CD57, NKG2A and NKG2C. (A) Gating of mononuclear cells, minus the debris, (B) Gating of lymphocytes, (C) Gating $CD56^{+}$ cells from the lymphocyte gate, (D) Gating of $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cells, (E) Gating of NKG2A and NKG2C within the $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cell subsets, (F) Gating of $NKG2A^{+}CD57^{+}$ expression within the $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cell populations, (G) Gating of $NKG2C^{+}CD57^{+}$ expression within the $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cell populations.

The following gating strategy was undertaken as shown in Figure 2.29 to identify and distinguish Regulatory T (CD3+ CD4+CD25+CD127lo FoxP3+) cells via intranuclear staining within healthy control and patient PBMCs.

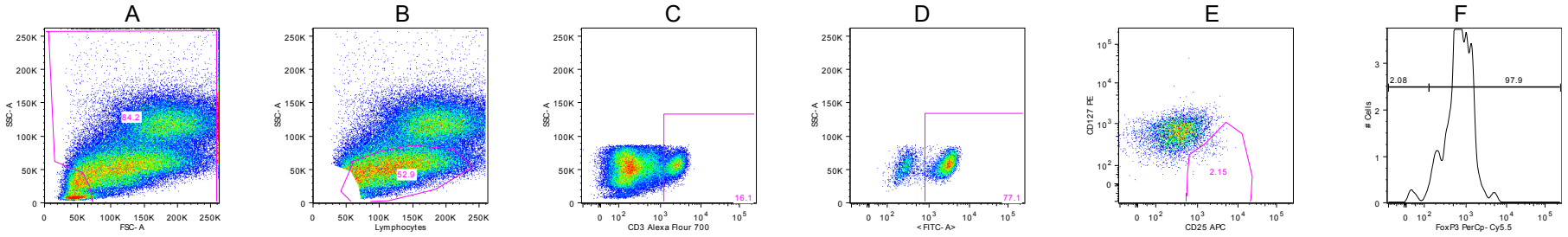


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Figure 2.29 Gating strategy for Regulatory T cells. Thawed healthy control and patient PBMCs were stained with monoclonal antibodies for surface expression of CD3, CD4, CD25, CD127 and FoxP3. (A) Gating of mononuclear cells, minus the debris, (B) Gating of lymphocytes, (C) Gating of $CD3^+$ T cells from the lymphocyte gate, (D) Gating of $CD4^+$ T cells, (E) Gating of $CD25^+$ $CD127^{lo}$, (F) Gating of FoxP3 within the $CD4^+CD25^+CD127^{low}$ T cell population.

2.8.5 NK cell isolation from thawed cell samples

NK cell isolation was performed on thawed healthy control PBMCs and patient PBMCs. The negative selection procedure was performed using the NK Cell Isolation kit II (Miltenyi, Germany). Once the NK cells were isolated they were centrifuged at 1500 rpm for 10 minutes and suspended in RPMI containing 10% v/v FBS. The purity of the isolated NK cells was then determined via flow cytometry as $CD3^{-}CD56^{+}$ cells.

2.8.6 Assessment of NK cell purity

The following gating strategy (Figure 2.30) was performed to identify the purity of NK cells after NK cell isolation. The mean purity (%) ± SD of all isolations carried out was 93.6 ± 3.42. NK cell functional studies could only be performed if there were more than 90% of NK cells within the DAPI gate.

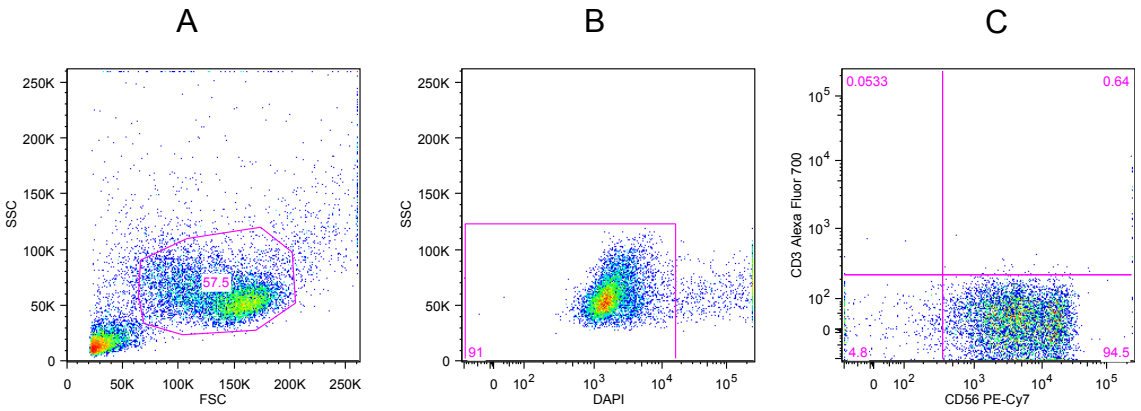


Figure 2.30 Gating strategy to determine the purity of isolated NK cells. The purity of isolated NK cells was confirmed by flow cytometry. (A) Gating of lymphocytes according to the forward (FSC) and side scatter (SSC). (B) Live cells were gated for using the DAPI stain. (C) Gating of NK cells ($CD3^{-}CD56^{+}$) from the live gate.

2.8.7 NK cell culture

NK cells from CBMCs, healthy adult and patient PBMCs were cultured overnight with IL-2. Protocols devised by Alnabhan *et al.* and Luevano *et al.* were followed. Table 2.19 describes the concentrations used for the respective NK cells (Alnabhan et al., 2015, Luevano et al., 2012).

Cytokine	PB NK cells	CB NK cells
IL-2	200 IU/mL (Chiossone et al., 2007)	1000 IU/mL (Condiotti et al., 2001)

Table 2.19 IL-2 Concentrations for adult healthy control, CB and patient NK cells.

IL-2 was added to promote NK cell expansion and proliferation. Cell culture media was prepared by adding 1% Penicillin-Streptomycin, 10% v/v FBS to RPMI 1640. Lyophilised human recombinant IL-2 was reconstituted in PBS supplemented with 0.1% w/v BSA. IL-2 was prepared and diluted in culture media before use. A volume of 1 mL of media containing IL-2 was added to $1x10^6$ NK cells. The cells were then cultured in 96 well-round bottom plates at 37^oC, 5% CO_2 overnight before use.

2.8.8 NK cell cytotoxicity

NK cell cytotoxicity was investigated after NK cells from healthy controls patient PBMCs were incubated overnight. K562 target cell lines were used for this assay. These cells are HLA-deficient leukaemic cells and were sub-cultured prior to use within 20% v/v FBS culture media and used for the assay when they had reached a log phase of 0.5×10^6 / mL. K562 cells were then washed and labelled with a PKH67 Green Fluorescent Linker Kit (Sigma). K562 cells were labelled as per the manufacturer's instructions. Fluorescently labelled K562 cells were washed and co-cultured with NK cells at a 1:1 ratio and incubated at 37^oC, 5% CO_2 for 4 hours. Cytotoxicity was measured via flow cytometry.

2.8.9 Assessment of NK cell cytotoxicity via flow cytometry

The following gating strategy shown in Figure 2.31 was used to identify the cytotoxic capacity of NK cells, isolated from patient samples. Measuring the percentage reduction in the K562 cell population assessed the NK cell cytotoxicity. The percentage reduction of K562 cells was calculated where there was a reduction in the populations seen within figure 2.31, B (ii) and (iii). Respectively, this was compared to a complete K562 population that was not incubated with patient NK cells.

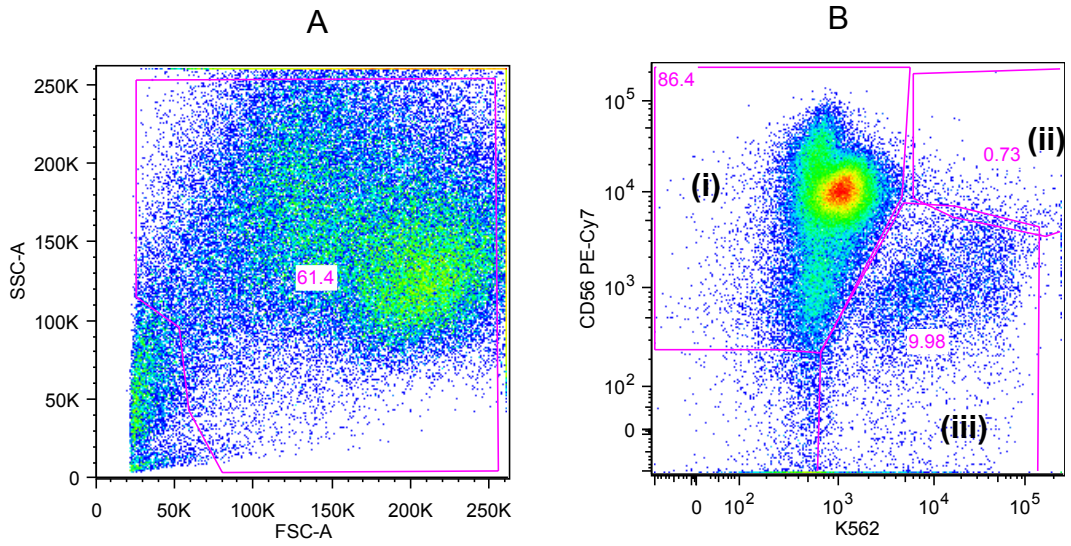


Figure 2.31 Gating Strategy to determine the reduction in K562 cells after incubation with NK cells. NK cell cytotoxicity was assed via the percentage decrease in the K562 population. (A) Gating of co-cultured NK cells and K562 cells. (B) Gating of (i) CD56+ NK cells, (ii) K562 Cells and conjugated CD56+ and (iii) K562 cells.

2.8.10 Statistical analysis

Statistical comparisons were performed with GraphPad Prism software using non-parametric, Mann-Whitney test. Results are presented as tukey plots and p values of <0.05 (*), <0.01(**), <0.001 (***) and <0.0001 (****) were considered statistically significant. Furthermore, overall survival was evaluated with Kaplan-Meier estimates.

Chapter 3 : Investigating the kinetics and diversity of immune cell reconstitution in cord blood transplant patients

3.1 Background and Aim

HSCT is associated with delayed immune reconstitution and the kinetics of reconstitution differs between graft types. Delayed immune reconstitution can persist for up to two years post-transplant, with an increased risk of infection, relapse and secondary malignancy (Ogonek et al., 2016, Gallagher and Forrest, 2007, Rizzo et al., 2009). The speed of immune reconstitution post-HSCT is crucial as it could reduce the risk of infection and prevent disease relapse. Furthermore, studies have been performed in BM and mPB transplanted patients to understand whether the kinetics of immune reconstitution impacts post-transplant outcomes (Seggewiss and Einsele, 2010, Bosch et al., 2012, Peggs and Mackinnon, 2004, Fry and Mackall, 2005, Geddes and Storek, 2007).

Immune reconstitution is delayed in CBT recipients compared to BM and mPB transplanted patients. The reconstitution of immune cells could be delayed by variables such as: HLA-disparity between the host and the donor; types of conditioning (chemotherapy/radiation regimen) used; depletion of T cells within the graft; GvHD and low TNC count within the graft (Lucchini et al., 2015). Measuring the kinetics of reconstitution could provide a time frame of cellular reconstitution post-transplant.

In HSCT, time to neutrophil and platelet engraftment has been used as a predictive factor for outcomes post-transplant. Neutrophil and platelet engraftment has been measured in CBT patients and is delayed when compared to BM and mPB recipients (Barker et al., 2015, Barker et al., 2005, Scaradavou et al., 2013). It has been further reported that the delayed engraftment leads to higher morbidity and mortality (Komanduri et al., 2007).

In CBT, identifying the kinetics of reconstitution of major immune subsets will provide a better understanding of their recovery post-transplant. This includes cells such as: CD45+ cells, CD14+ monocytes, CD3+ T cells, CD4+ T cells, CD8+ T cells and CD19+ B cells. The importance of these cells is described below.

All haematopoietic cells except erythrocytes and platelets express CD45, which is transmembrane glycoprotein known as the common leucocyte antigen (CLA). CD45 is abundantly expressed on the surface of leucocytes such as B cells, NK cells, monocytes and T cells (Hermiston et al., 2003). With this in mind, the absolute counts of CD45+ cells have been measured in this current study to gain an insight into the kinetics of leucocyte reconstitution in CBT patients. Furthermore, the expression patterns of CD45 have also been used as a laboratory measure to identify and quantify the absolute levels of leucocytes within the patient samples. Additionally, the use of CD45 has allowed sub-analysis of lymphocytes, monocytes and granulocytes within patient samples as detailed in Chapters 4, 5 and 6.

In healthy individuals, monocytes are believed to have an important role at restoring injured tissues and controlling inflammatory responses. Monocytes express CD14 on their surface, which is a phospholipid anchored membrane protein. (Biswas and Mantovani, 2010, Thoma et al., 2012). BMT and mPB transplant studies have shown that CD14+ monocytes are one of the first immune cell types to recover post-transplant. Furthermore, reconstitution of monocytes occurs within one month post-transplant (Tayebi et al., 2001, Maris et al., 2003, Storek et al., 2001a). However, patterns of monocyte reconstitution within CBT patients are poorly defined and therefore have been investigated in this study.

In adaptive immunity, CD19+ B cells are one of the vital immune cells involved in humoral immune responses. In a study analysing 133 paediatric and adult CBT patients, B cell reconstitution was shown to be quicker as compared to mPB and BM recipients (Nakatani et al., 2014), reaching normal healthy ranges

by a median time of six months. Therefore, measuring the kinetics of CD19+ B cell reconstitution will provide information into the restoration of humoral immunity. The overall association of CD19+ B cell reconstitution and outcomes post-CBT has not been clearly defined. However, it has been shown that CBT patients with higher absolute numbers of B cells post-transplant have a reduced incidence of cGvHD (Beaudette-Zlatanova et al., 2013).

In CBT studies, T cell reconstitution is delayed compared to BM and mPB recipients (Moretta et al., 2001b, Renard et al., 2011, Jacobson et al., 2012, Bartelink et al., 2013, Charrier et al., 2013, Clave et al., 2013, Oshrine et al., 2013). Komanduri *et al.* demonstrates that T cell reconstitution is delayed in CBT patients and believes that early B cell and NK cell reconstitution compensates this. Jacobson *et al.* also shows that T cell reconstitution is delayed for up to one year post-CBT and B cell reconstitution takes place within six months post-CBT (Jacobson et al., 2012, Komanduri et al., 2007). The patterns of T cell reconstitution varies between CBT studies and this is due to the fact that there are differences in conditioning regimens, varying recipient ages and certain patients are administered T-cell depletion. Additionally, as CB grafts have lower T cell numbers compared to mPB and BM grafts, it is believed that this delays T cell reconstitution in CBT patients. (Szabolcs and Niedzwiecki, 2008).

T cells are also vital cells involved in adaptive immune responses and are essential inducers of cell-mediated effects to clear infection. However, in HSCT T cells are also involved in GvHD development (Jadus and Wepsic, 1992). Rapid reconstitution of T cells could lead to peripheral expansion of T cells and subsequently lead to the development of aGvHD. Understanding the reconstitution patterns of T cells in CBT studies provides a deeper insight into how these cells may be able to control infection or whether they could be actively involved in GvHD reactions. Additionally, T cells are known to play a role in GvL, as previously described (Barrett, 2008). Therefore, monitoring the patterns of T cell reconstitution could give an insight as to whether there is an optimised GvL effect taking place in CBT patients post-transplant.

Reconstitution of T cells in CBT patients is partially dependent on the survival of adoptively transferred T cells that come from the CB graft. Patients are also reliant on the *de novo* production of T cells from the thymus for complete T cell reconstitution (Douek et al., 1998, Douek et al., 2000, Lewin et al., 2002, Poulin et al., 1999). T cells exclusively express a multimeric protein on their surface known as CD3. This is one of the surface receptors involved in activation of cytotoxic T cells and T helper cells. Polypeptide chains within the CD3 protein associate with the T cell receptor (TCR), which leads to the activation of the T cell (Guy and Vignali, 2009). Monitoring the expression of CD3+ T cells within this current study will provide an understanding of the overall kinetics of T cell reconstitution within CBT patients.

T cells mature within the thymus into two types, CD4+ and CD8+ T cells. CD4+ T cells are also known as T helper cells, which express the CD4 glycoprotein on the surface of the cell. T helper cells play a vital role in adaptive immune responses and mediate cytotoxicity of infected cells via cytokine release (Gill et al., 2003). Important roles of CD4+ T cells in immunity are well illustrated in HIV/AIDS infected patients. These patients are immunodeficient due to the loss of CD4+ T cells. This can lead to the development of fatal opportunistic infections that cannot be cleared. Similarly, CBT patients are immunocompromised and have reduced numbers of CD4+ T cells within the periphery, which contributes to the development of opportunistic infections (Zajac et al., 1998). In HSCT, delayed reconstitution of CD4+ T cells increases the risk of transplanted related mortality and decreases overall survival of allogeneic HSCT recipients from conventional donors and sUCBT in paediatric patients. Therefore, this highlights a key role for CD4+ T cells that may be required in CBT recipients (Admiraal et al., 2015, Bartelink et al., 2013, Berger et al., 2008).

Moreover, CD8+ T cells are cytotoxic T cells and are important for immune defences against intracellular pathogens such as viruses, bacteria and for tumour surveillance. CD8 receptors are a dimeric co-receptor expressed on CD8+ T cells (cytotoxic T cells). The co-receptor is composed of a CD8α and CD8β chain. CD8+ T cells recognise peptides presented by class I HLA

molecules, expressed on all nucleated cells. The CD8 α chain interacts with the α3 portion of the class I MHC molecule and this interaction keeps the TCR and the target cell closely bound together. The CD8 receptor also plays a fundamental role in T cell signalling, where the cytoplasmic tails of the receptor interact with lymphocyte-specific protein tyrosine kinase (Lck). In turn, this initiates phosphorylation of the CD3 receptor and chains of the TCR complex. Subsequently, this leads to activation of transcription factors such as NFAT, NF-κB and AP-1. This leads to cytotoxicity of the target cell (Andersen et al., 2006, Artyomov et al., 2010, Smith-Garvin et al., 2009).

Therefore, CD3, CD4 and CD8 have therefore been included as important T cell markers within this study. Measurement of the respective surface markers will provide information as to whether patients are able to restore the overall T cell population, T helper cell population and the cytotoxic T cell population post-transplant.

The Immune Reconstitution Study (IRES-CBT) REC number: 09/H0706/35 was designed and set up in 2009 to measure reconstitution of the main immune subsets in CBT patient samples. Patient samples were initially collected and processed by Dr Sameer Tulpule between 2009 and 2010. Dr Damini Tewari then followed this up between 2010 and 2011. I then processed patient samples from the 1st of May 2012. The patient sample collection and processing was kept uniform throughout the study.

Taken together and considering the importance of the aforementioned cells, this current study investigated the diversity and kinetics of immune cells such as leucocytes, monocytes, B cells and T cells in CBT patients. The aims of this chapter are to investigate the following:

1. Map the kinetics of recovery of CD45+ cells in CBT patients.
2. Map the kinetics of recovery of CD14+ monocytes in CBT patients.
3. Map the kinetics of CD19+ B cells in CBT patients.
4. Map the kinetics of CD4+ and CD8+ T cells in CBT patients.
5. Map the time to neutrophil and platelet engraftment in CBT patients.
6. Determine the overall survival of CBT patients.

3.2 Diversity and kinetics of immune recovery in cord blood transplant patients

A total of 67 adult patients were included in this study recruited from 13 hospitals across the UK, as described in Chapter 2, section 2.1.3. The characteristics of the patients included in this study are listed in Table 3.1.

		Count	Total number of patients (%)	Median	Range
Gender	Female	24	36		
	Male	43	64		
Age (years)				48	21 - 70
Diagnosis	Acute Leukaemia	38	57		
	Myelodysplastic syndrome/myelodysplasia (MDS)	11	16		
	Myeloproliferative disorder (MPD)	3	4		
	Lymphoproliferative disorder	13	19		
	Bone marrow failure syndrome	2	3		
Status at Transplant	Complete remission (CR)	44	66		
	Partial remission (PR)	6	9		
	Relapse/ Progression	6	9		
	Stable/ Untreated	5	7		
	Missing	6	9		
CMV (IgG)	Negative	19	28		
	Positive	43	64		
	Missing	5	7		
ABO	A	22	33		
	B	7	10		
	AB	1	1		
	O	28	42		
	Missing	9	13		
Conditioning	MAC	17	25		
	RIC	50	75		
HLA Match	3/6	3	4		
	4/6	29	43		
	5/6	19	28		
	6/6	5	7		
	Missing	11	16		
Graft type	Single CBU	3	4		
	Double CBU	64	96		
Survival (months)				19	1 - 24

Table 3.1 Characteristics of patients enrolled onto the immune reconstitution study.

3.2.1 CD45+ cell count recovery following allogeneic cord blood transplantation

CD45+ cells were quantified within CBT patients to better understand the overall leucocyte cell reconstitution post-transplant. CD45 is expressed on leucocyte cells and quantification of CD45+ leucocyte cells is poorly defined in CBT studies. CD45+ cells were quantified as cells per microliter and represent absolute counts of CD45+ leucocyte cells in CBT patients, as seen in Figure 3.1.

In CBT patient samples, the median absolute count (3710 cells/µl) of CD45+ cells at day 28, were lower compared to healthy control PB (5987 cells/µl). Respectively, at 28 days post-transplant, the interquartile range for patients (1302 - 8090 cells/µl) was lower compared to healthy control PB (4825 - 7568 cells/µl). In comparison to the successive time points and healthy control PB, CD45+ cell counts were lowest in CBT patients at 28 days post-transplant. At subsequent time points the median absolute count of CD45+ cells increased in CBT patients and the interquartile ranges also showed to be broader compared to healthy control PB. There was a continued increase in the median absolute counts of CD45+ cells between 28 days and 180 days post-CBT. From 60 days to 720 days post-CBT, the CD45+ median absolute counts were within the same range as the median absolute counts measured in healthy control PB, indicating that patients had a rapid reconstitution of CD45+ cells by 60 days post-CBT. Absolute counts of CD45+ cells increased at 60 days post-CBT where median levels (8186 cells/µl) were higher compared to healthy control PB (5987 cells/µl). However, due to the interquartile ranges being within the healthy control PB range, no statistical significance was observed. This indicates that patients have complete reconstitution of CD45+ cells by 60 days post-CBT.

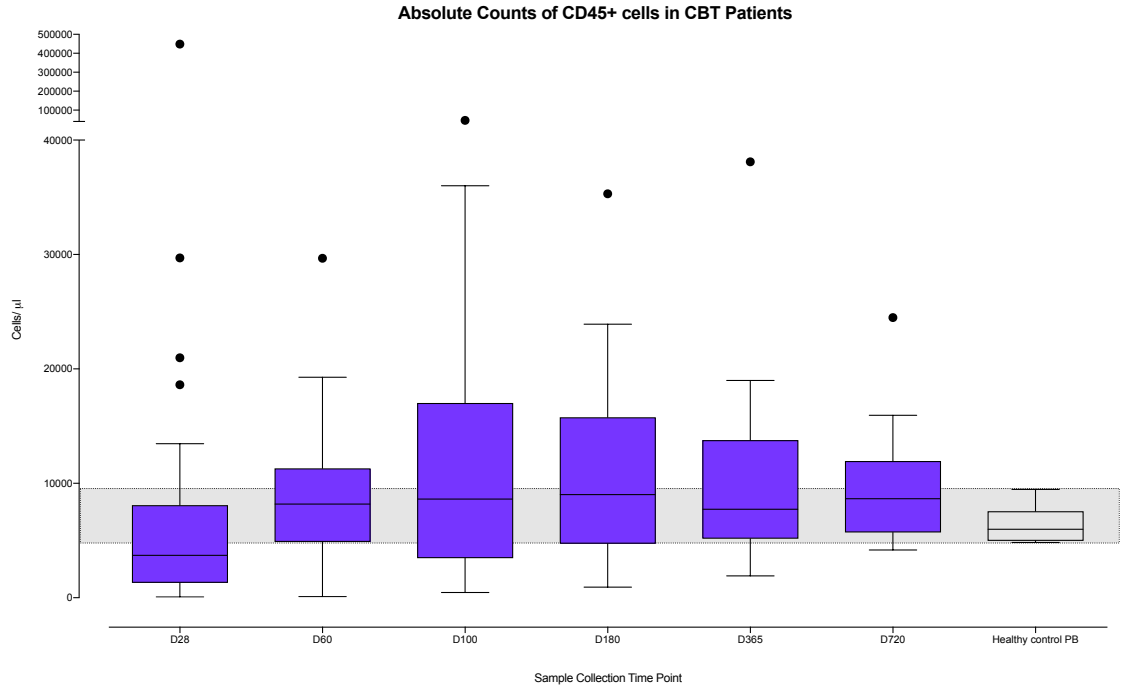


Figure 3.1 Absolute counts of CD45+ leucocytes in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter of CD45+ leucocytes within CBT patients in the UK. Flow cytometry was performed to quantify CD45+ cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as tukey analysis. No significance was observed.

3.2.2 CD14+ monocyte count recovery following allogeneic cord blood transplantation

Monocytes are involved in innate immune responses and are one of the first subset of cells recruited to sites of tissue injury and inflammatory sites. The reconstitution of monocytes is not well studied in CBT patients and quantification of monocytes in this current study will provide an insight in to their reconstitution after CBT. Monocytes (CD14+) were identified and quantified by flow cytometry and patterns of monocyte reconstitution within CBT patients (Figure 3.2) are compared to levels observed in healthy control PB. Reconstitution of CD14+ monocytes took place by 28 days post-CBT. In CBT patients, there was a greater range of CD14+ monocyte levels post-transplant in comparison to healthy control PB.

Median absolute levels of monocytes were higher throughout all the time points studied compared to healthy control PB. CD14+ monocyte absolute counts increased between 28 and 180 days post-CBT. At 180 days post-CBT, the absolute counts of CD14+ monocytes were significantly higher compared to healthy control PB ($p = 0.03$). CD14+ monocyte absolute counts in patients remained higher between 180 and 720 days post-transplant compared to healthy control PB. The median absolute counts were 873 cells/μl and 741 cells/μl at day 365 and day 720, respectively, which were significantly higher compared to healthy control PB ($p=0.005$ and $p=0.03$, respectively). The data therefore suggests that there is reconstitution of CD14+ monocytes within the PB of patients by 28 days post-CBT.

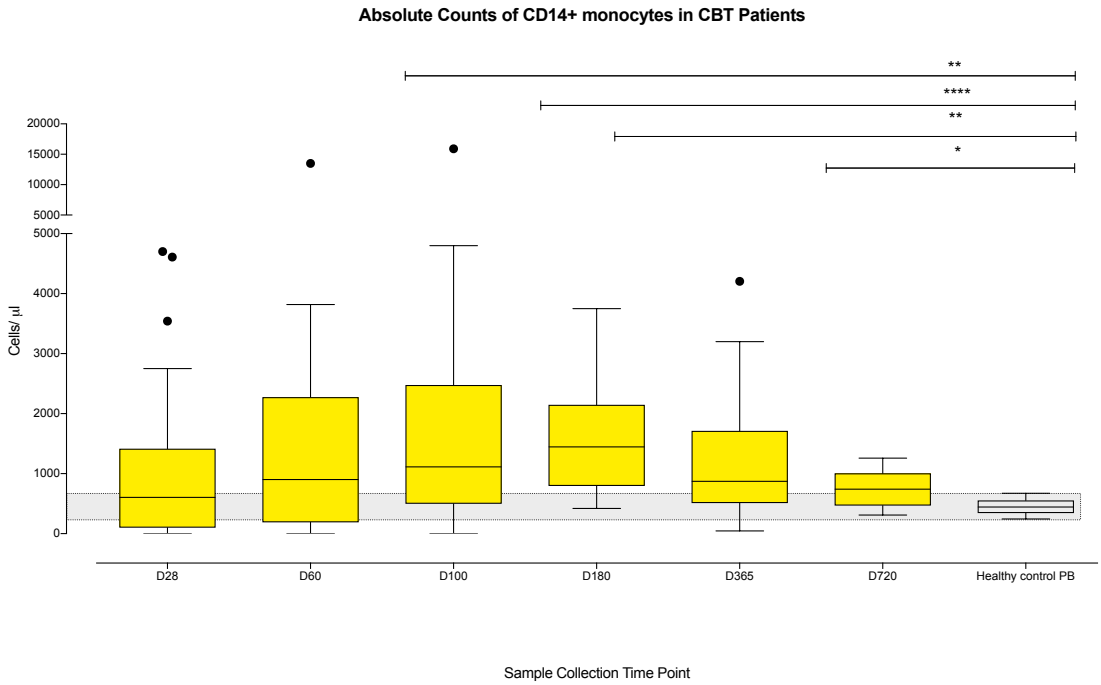


Figure 3.2 Absolute counts of CD14+ monocytes in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD14+ monocytes cells within CBT patients in the UK. Flow cytometry was performed to quantify CD14+ monocytes. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as tukey analysis, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).

3.2.3 CD3+ T cell recovery following allogeneic cord blood transplantation

CD3 expression levels were analysed within this study as it is a multimeric protein complex found on all T cells and is an important co-receptor that binds non-covalently with the T cell receptor (Smith-Garvin et al., 2009). The CD3 molecule is primarily expressed on T cells therefore making it a key cell surface marker to monitor T cell reconstitution. In various CBT studies, it has been shown that T cells have a delayed pattern of reconstitution compared to BM and mPB transplant (Jacobson et al., 2012, Komanduri et al., 2007, Cohen et al., 2006). By monitoring the reconstitution patterns of CD3+ T cells within this current study, the broad recovery of T cells within CBT patients has been distinguished. Levels of CD3+ T cells can be seen in Figure 3.3.

At 28 days post-transplant, median absolute counts of CD3+ T cells within patients (138 cells/μl) were significantly lower compared to healthy control PB (1475 cells/μl) ($p=0.0001$). The absolute counts of CD3+ T cells increased at 60 days in CBT patients. However, the interquartile range and median absolute levels remained below the healthy control PB interquartile range. At day 100, median absolute counts (528 cells/μl) of CD3+ T cells were significantly lower compared to healthy control PB (1475 cells/μl) ($p=0.005$). At 365 and 720 days post-CBT, the absolute counts of CD3+ T cells were lower compared to healthy control PB. However, the upper quartile of the interquartile ranges at the respective time points reached the interquartile range seen within healthy control PB. The data therefore highlights that the absolute numbers of CD3+ T cells increases over the two year follow up period, post-CBT. However, the median absolute counts at all times points were lower in CBT patients compared to healthy control PB. Overall, this indicates that there is a delay in CD3+ T cell reconstitution in CBT patients post-transplant.

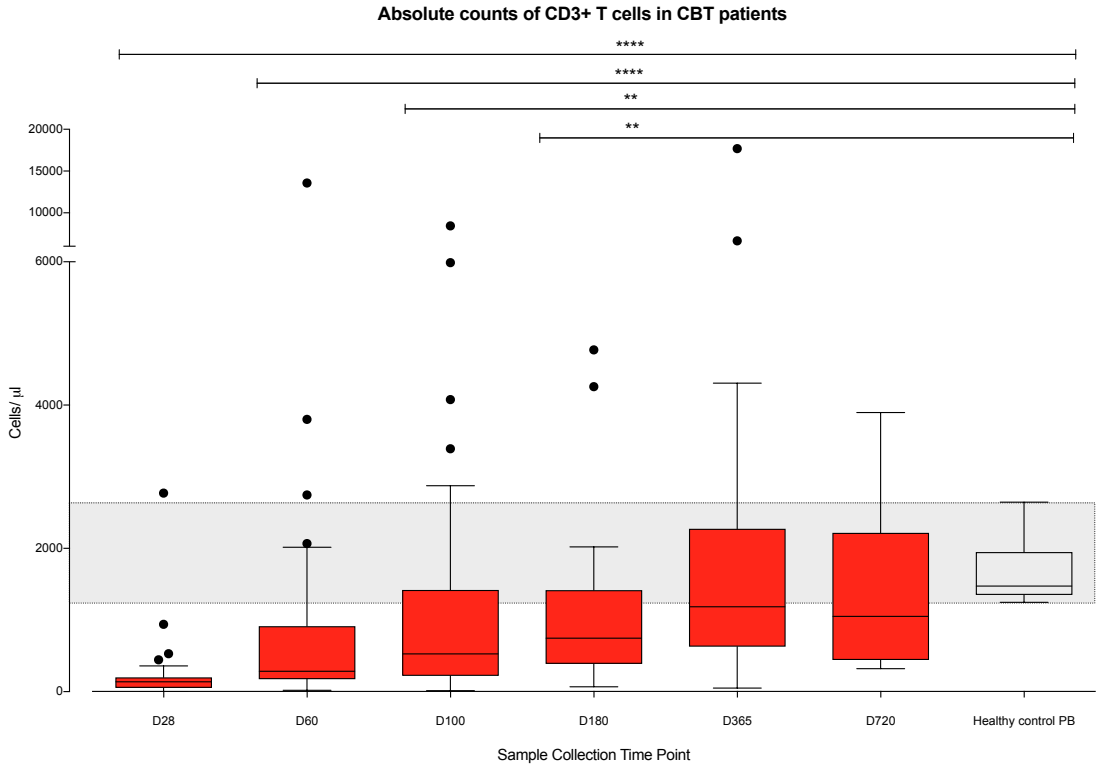


Figure 3.3 Absolute counts of CD3+ T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD3+ T cells, in CBT patients in the UK. Flow cytometry was performed to quantify CD3+ T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as tukey analysis, $p<0.05$ (*), $p<0.01$ (**) and $p<0.0001$ (****).

3.2.4 CD4+ T cell count recovery following allogeneic cord blood transplantation

The kinetics of recovery of CD4+ T cells was investigated within CBT patients, as CD4+ T cells are key mediators that clear pathogenic infection (Sant and McMichael, 2012). T cells also play a fundamental role in GvHD development and delayed reconstitution of T cells could be associated with the incidence of relapse. With these important challenges faced, it is important to define the time in which CD4+ T cell reconstitution takes place post-CBT. This will aid in defining whether T cells influence post transplant outcomes. Levels of CD4+ absolute counts can be seen in Figure 3.4.

At 28 days post-CBT, median absolute count of CD4+ T cells (92 cells/μl) were significantly lower compared to healthy control PB (864 cells/μl) and CBT patients had a significantly narrower interquartile range (35-159 cells/μl) of CD4+ T cells compared to healthy control PB (729 - 1174 cells/μl) ($p=0.0001$). At 60 days post-CBT, median absolute counts of CD4+ T cells (188 cells/μl) were significantly lower compared to healthy control PB with a significantly narrower interquartile range in patients (129-229 cells/μl) ($p=0.0001$) compared to healthy control PB. At successive time points between 100 and 720 days post-CBT, the median CD4+ T cell absolute counts increased. However, they remained significantly lower compared to healthy control PB. The broadest interquartile ranges were recorded in CBT patients at 365 days (343-898 cells/μl) and 720 days (310-981 cells/μl) post-transplant. Overall, the CD4+ T cell data suggests that there is a gradual increase in absolute numbers of CD4+ T cells between 28 and 720 days post-CBT. However, the median absolute cell levels remained below the median levels in healthy controls PB for up to 720 days post-transplant. This data therefore suggests that CD4+ T cell reconstitution is delayed in CBT patients and could take longer than 720 days to redevelop post-transplant.

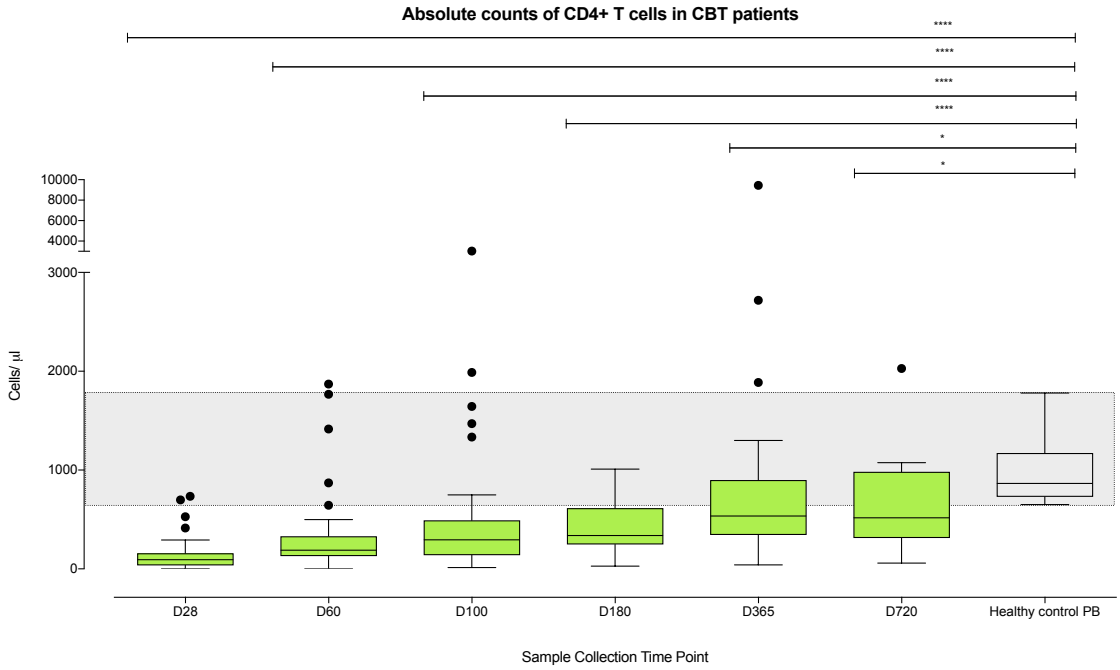


Figure 3.4 Absolute counts of CD4+ T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD4+ T cells within CBT patients in the UK. Flow cytometry was performed to quantify CD4+ T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as tukey analysis, $p<0.05$ (*) and $p<0.0001$ (****).

3.2.5 CD8+ T cell count recovery following allogeneic cord blood transplantation

CD8+ T cells play a pivotal role in clearing fungal and viral infections (Andersen et al., 2006). In HSCT, CD8+ T cells have been shown to play a key role in the clearance of infection within the first 100 days of transplant (Montoro et al., 2016, Cahu et al., 2009). Along with infection and their cytotoxic capacity, understanding CD8+ T cell reconstitution could also help determine whether they play a role in GvHD development or reduce the incidence of relapse in CBT patients. Overall, the absolute count of CD8+ T cells was measured in this study to define the time in which CD8+ T cells reconstitute in CBT patients.

At 28 days post-CBT, absolute counts of CD8+ T cells, shown in Figure 3.5, were significantly lower compared to healthy control PB ($p=0.0001$). Respectively, at 28 days post-CBT, the interquartile range (12-50 cells/µl) was lowest compared to healthy control PB (442-898 cells/µl). Absolute counts of CD8+ T cells increased at 60 days post-transplant and continued to increase up to 365 days. At 365 days, the absolute count of CD8+ T cells was similar to healthy control PB. Furthermore, at 365 days post-CBT, the interquartile range was broader (254-1500 cells/µl) compared to healthy control PB (442-898 cells/µl). Overall, this data suggests that there is a gradual increase in the absolute number of CD8+ T cells between 28 days and 365 days post-transplant. Also, there is reconstitution of CD8+ T cell counts within the peripheral blood of patients by 365 days post-CBT.

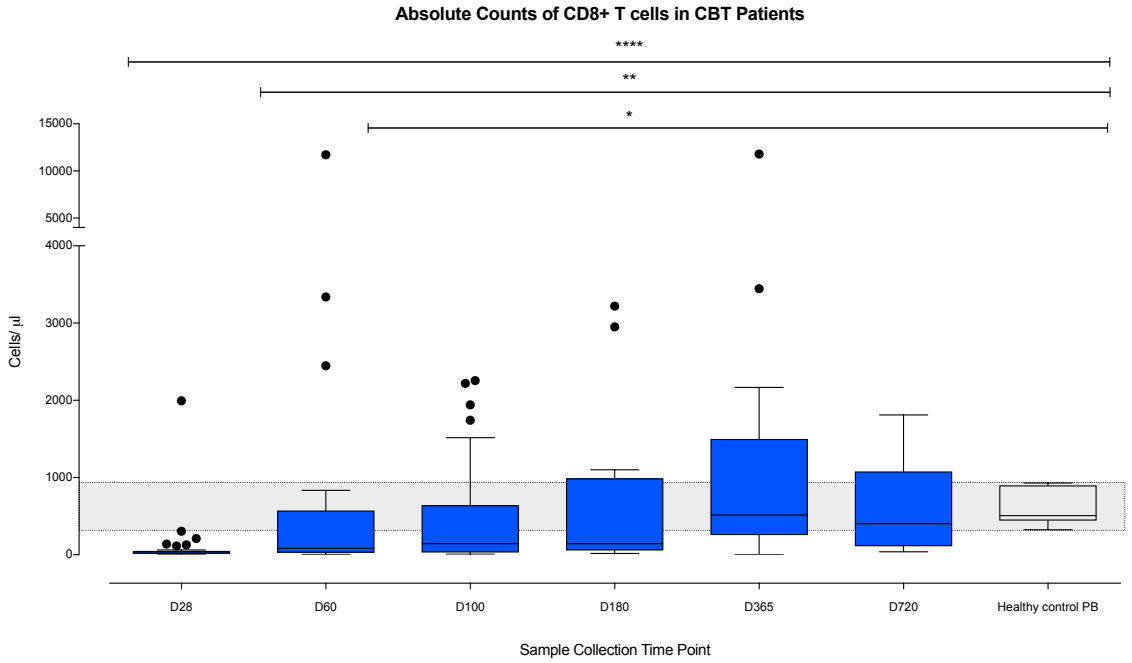


Figure 3.5 Absolute counts of CD8+ T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD8+ T cells within CBT patients in the UK. Flow cytometry was performed to quantify CD8+ T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as tukey analysis, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).

3.2.6 CD19+ B cell count recovery following allogeneic cord blood transplantation

In allogeneic HSCT studies, it has been demonstrated that B cells have a different pattern of reconstitution compared to T cells (Bemark et al., 2012, Ogonek et al., 2016). Furthermore, in CBT there is a normalization of CD19+ B cells counts by six months, which is faster compared to BM and mPB transplant. It has also been shown in other studies that early B cell reconstitution is also associated with reduced incidence of cGVHD (Beaudette-Zlatanova et al., 2013). Overall, CD19+ B cells are known to play a fundamental role in adaptive immune responses and as CBT patients are prone to several opportunistic infections, B cells are highly required to protect and clear them. These fundamental roles post-transplant have led to us to determine the kinetics of reconstitution of CD19+ B cells in CBT patients.

As shown in Figure 3.6, at 28 days post-transplant median absolute counts of CD19+ B cells (6 cells/μl) was significantly lower compared to healthy control PB (302 cells/μl) ($p=0.0001$). At 60 days post-CBT, there was an increase in the absolute counts of CD19+ B cells in CBT patients and the interquartile range was also seen to broaden. Subsequently, at day 100, there was a further increase in the CD19+ B cell counts within patients and the interquartile range (63 – 1211 cells/μl) was broader compared to healthy control PB (194 – 356 cells/μl). Median absolute levels of CD19+ B cells increased in CBT patients (997 cells/μl) at day 180 and were significantly higher compared to healthy control PB (302 cells/μl) ($p=0.01$). The interquartile range (307 – 2403 cells/μl) was broadest in CBT patients at day 180 compared to all the respective time points and was broader compared to healthy control PB (194 – 356 cells/μl). Absolute counts of CD19+ B cells and the interquartile range remained higher compared to healthy control PB at 365 and 720 days post-CBT ($p=0.0002$ and $p=0.0007$, respectively). This data suggest that the reconstitution of CD19+ B cells takes place by 100 days post-CBT. The absolute number of CD19+ B cells increased between 100 days and 365 days post-transplant and exceeded absolute counts observed within healthy control PB.

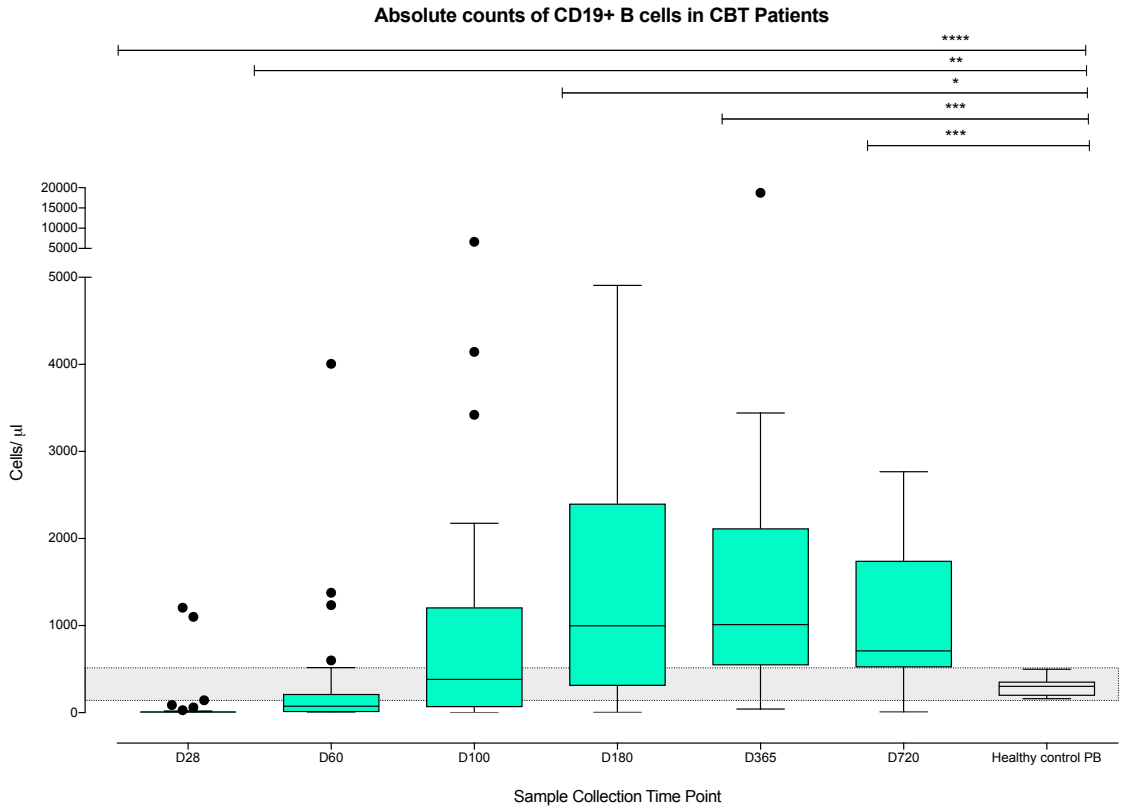


Figure 3.6 Absolute counts of CD19+ B cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD19+ B cells within CBT patients in the UK. Flow cytometry was performed to quantify CD19+ B cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as tukey analysis, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).

3.2.7 Engraftment following cord blood transplantation

3.2.7.1 Neutrophil engraftment following cord blood transplantation

Time to neutrophil engraftment was measured, at the respective transplant centres, in patients as three consecutive days at which they had a neutrophil count greater than 0.5 x 10^9/L. The data was retrieved as described in Chapter 2, section 2.3.11. 62 patients (93% of the total patient group) had neutrophil count greater than 0.5 x 10^9/L. This was achieved for all 62 patients by 56 days post-transplant. The median time to neutrophil engraftment was 20 days post-transplant. The range of neutrophil engraftment was 5-56 days post-transplant. For five patients, time to neutrophil engraftment data was unavailable and these five patients have been excluded from this analysis.

3.2.7.2 Platelet engraftment following cord blood transplantation

Time to platelet engraftment was measured in CBT patients; at their respective transplant centres, as three consecutive days at which they had a platelet engraftment count greater than 20.0 x 10^9/L. The data was retrieved as described in Chapter 2, section 2.3.11. Platelet engraftment occurred within 57 patients (85% of the total patient group). Time to platelet engraftment for all the patients was 120 days post-transplant. The median platelet engraftment was 40 days post-transplant. The range of platelet engraftment in CBT patients was 1-118 days post-transplant. For ten patients, there was no data available for their platelet engraftment and these patients were excluded from this analysis. This data suggests that CBT patients have a median time to platelet engraftment within 40 days post-CBT and the time to platelet engraftment within all the CBT patients can take up to 118 days post-transplant.

3.2.8 Overall survival of patients after cord blood transplant

Overall survival data was retrieved as described in Chapter 2, section 2.3.11. Figure 3.9 shows the probability of survival after CBT in patients recruited on to the immune reconstitution study.

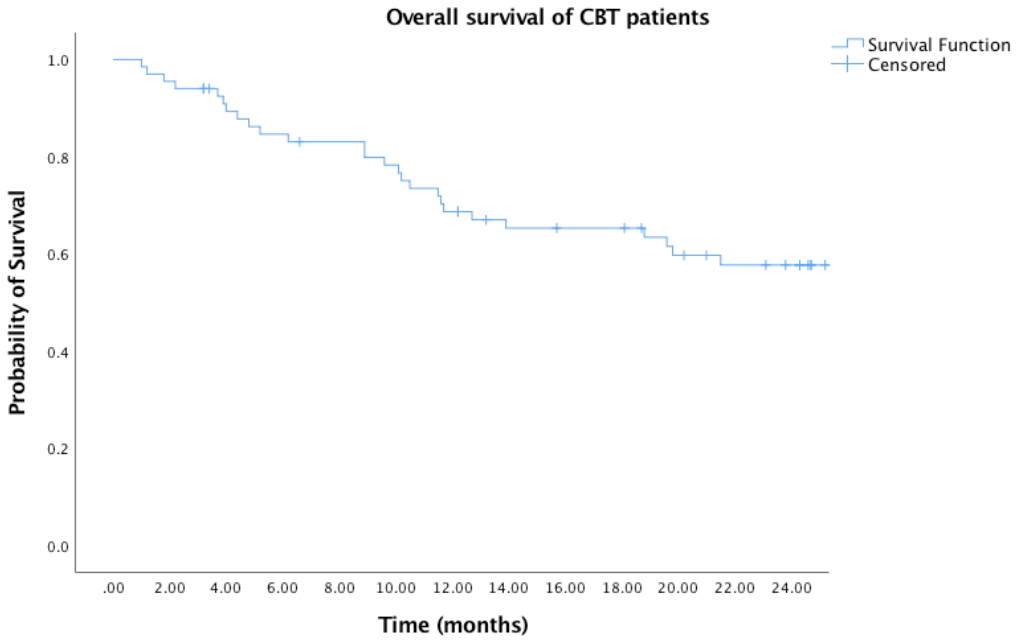


Figure 3.7 Overall survival in CBT patients. Kaplan-Meier curve for overall survival in CBT-patients up to 720 days post-transplant.

In this study, the probability of overall survival was 61% (95% Confidence interval (CI), 37-53%) at two years post-transplant. A total of 26 patients died (39% during the study (95% CI, 31-42%). 16 patients died of relapse or disease progression, eight patients died due to transplant related causes, two died due to unknown causes.

3.2.9 Summary of immune kinetics and diversity in cord blood transplant patients

The following timeline was constructed to give an overview of the kinetics and diversity of immune reconstitution in CBT patients within this current study. Figure 3.8 summarises immune kinetics and diversity data obtained in this study and shows the various time points at which immune cells reconstitute within CBT patients. It had been decided that a population had reconstituted, as the median range was no longer statistically significantly different from healthy controls. The summary of the results (Figure 3.8) shows the kinetics and diversity of immune cell reconstitution that takes places over two years post-transplant. Early reconstitution of CD14+ monocytes and CD45+ leucocytes took place by 60 days post-CBT. Furthermore, by day 60 there was complete neutrophil engraftment. Subsequently, CD19+ B cell reconstitution took place between 100 and 180 days post-CBT. At 120 days post-transplant, all CBT patients had complete platelet engraftment. T cell reconstitution occurred later with CD8+ T cell reconstitution occurring by day 365. However, CD3+ and CD4+ T cell absolute counts remained low for two years.

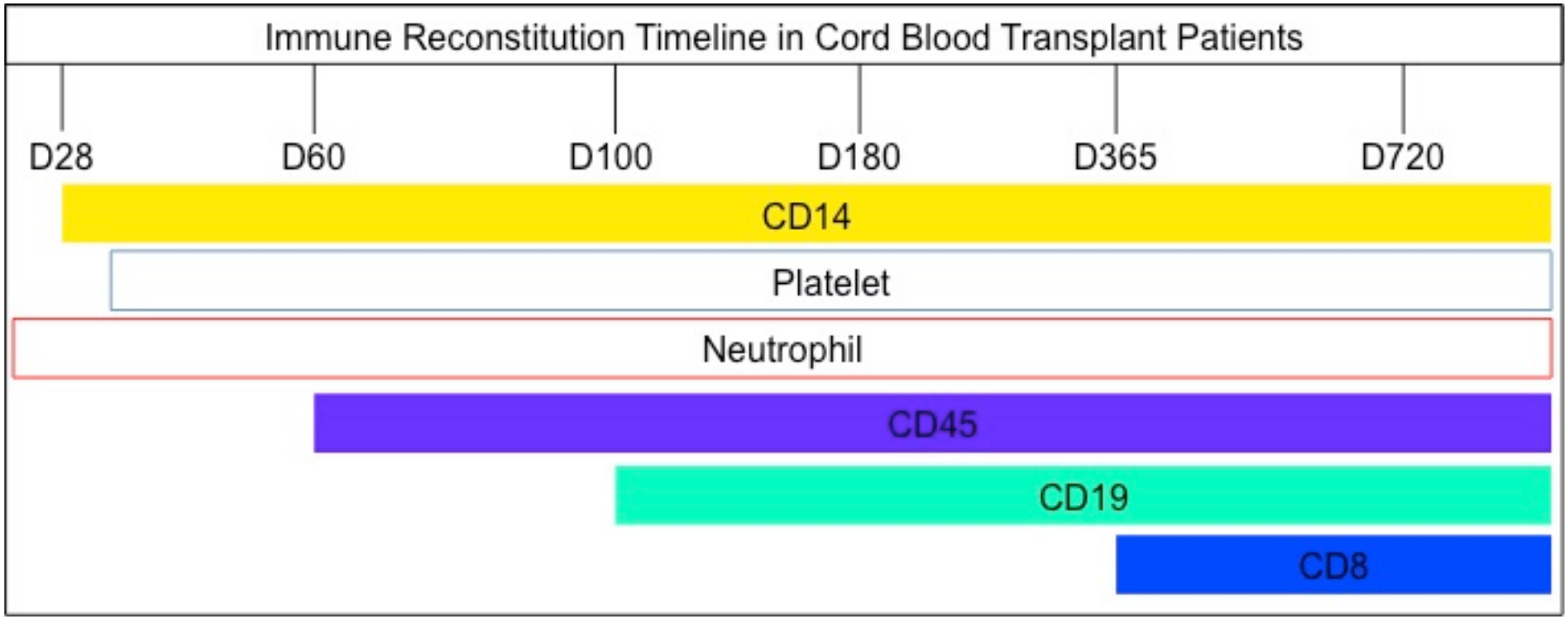


Figure 3.8 Summary of immune reconstitution kinetics and diversity in CBT patients. The figure above shows the various time points at which the various cellular subsets reconstitute within CBT patients. The figure summarises the data from all the absolute counts.

3.3 Discussion

The current study is the first study within the UK to investigate the diversity and kinetics of immune cells in CBT patients. To date, CBT is performed within the UK as a single or double transplant for adult patients. Conditioning protocols that have been used in this current cohort of patients are either RIC or MAC. RIC protocols used in the UK are devised from the Minneapolis RIC protocol (Flu, Cy, TBI CsA and MMF and ATG). However, MAC protocols within the UK can be chosen from two protocols – Minneapolis MAC protocol (Flu and Cy, TBI, CsA and MMF) or the Valencia MAC protocol (Flu, Thio, BU, ATG and CsA+MMF) (Shaw et al., 2009, Hough et al., 2016). For this current study, patients have not been divided into their respective conditioning groups due to the limited number of myeloablative conditioning patients enrolled on to the study. Therefore, all the data presented includes patients from both conditioning regimens.

The outcomes of CBT depend on the ability of the donor cells to migrate and engraft into the host without any hindrance (Zhang et al., 2003, Podesta, 2001). However, it is known that CBT patients have a delayed immune reconstitution post-transplant in comparison to BM and mPB transplanted patients (Beaudette-Zlatanova et al., 2013, Servais et al., 2014, Kanda et al., 2012). With this in mind, the current study was set up in 2009 to identify the reconstitution patterns of immune cell subsets in CBT patients.

Following conditioning and the infusion of the CBU, patients undergo an initial period of aplasia. Within this period of time, the donor CB-HSC will engraft in the patient and the engrafted cells will differentiate and proliferate within the BM. However, the time to neutrophil and platelet engraftment in CBT patients is delayed in comparison to BM and mPB transplant recipients (Seggewiss and Einsele, 2010, Petropoulou and Rocha, 2011). Seggewiss *et al.* showed that the median time to engraftment in BM, mPB and CB recipients is 21, 14 and 30 days, respectively(Seggewiss and Einsele, 2010). This highlights that engraftment is delayed in CBT recipients compared to BM and mPB recipients.

In this thesis, the time to neutrophil and platelet engraftment occurs earlier with a median time of 20 days and 40 days post transplant, respectively.

Additionally, It has been reported that the overall survival of CBT patients is similar to that of matched related donor (MRD) and matched unrelated donor (MUD) recipients with a range of 33 - 66% at 720 days post-transplant. (Brunstein et al., 2012, Chen et al., 2012, Ponce et al., 2011, Brunstein et al., 2010). Data from this current study also shows that CBT patients have an overall survival of 61% at 720 days post-transplant, which validates what has been documented in other studies.

In this current study, there is rapid reconstitution of CD45+ leucocytes and CD14+ monocytes by 60 days post-transplant. This corroborates data by Li *et al.* who also shows that CD14+ monocytes reconstitute rapidly and the absolute numbers of CD14+ monocytes remain high early post-transplant (Li et al., 2017). However, in the study conducted by Li *et al.* the reconstitution patterns of immune cells were recorded as early as seven days post-transplant, whereas in this current study the earliest reconstitution time point is 28 days post-transplant. This could mean that patients within our study could have normal CD45+ cell counts and CD14+ monocytic cell counts before 60 days post-CBT. It is therefore essential to add earlier time points between seven days and 28 days post-CBT in future immune reconstitution studies to better identify the kinetics and recovery of immune cell subsets in CBT patients. In this current project, blood samples were not collected before 28 days post-transplant, as routine blood tests required the collection of research blood samples after 28 days. Furthermore, patients are lymphopenic early post-transplant and accuracy in the counts would decrease, as there would be fewer subsets of cells. Additionally, patients could be unwell early post-transplant and could refuse sample donation.

In line with other studies, this project also shows that B cell reconstitution takes place early post-transplant, occuring between 100 and 180 days (Nakatani et al., 2014, Komanduri et al., 2007, Beaudette-Zlatanova et al., 2013). Furthermore, longitudinal studies of immune reconstitution in CBT recipients

show that absolute numbers of circulating B cells are higher in CBT patients compared to mPB and BM recipients (Beaudette-Zlatanova et al., 2013, Jacobson et al., 2012, Kanda et al., 2012).

Data from this current study compared to mPB grafts and BM grafts shows that the recovery of B cells is rapid post-transplant in CBT patients, shown in Table 3.2. There could be several reasons as to why B cells normalise faster within CBT patients compared to mPB or BMT recipients. Firstly, this could be due to a compensatory mechanism because of patients having significant T cell lymphopenia post-transplant. This has been demonstrated in HIV infected individuals where patients have deficiency of CD4+ T cells. However, this is compensated by expansion of CD19+ B cells (Malaspina et al., 2007, Malaspina et al., 2006). The second reason may be due to the fact that B cells arise from fetal stem cells within the liver and mature within the BM and not from adult stem cells. Once CB is infused into the patient, it is has been proposed that these B cells could expand from the fetal liver and induce rapid expansion of B cells within the recipient post-transplant. This gives rise to an expanded B cell population within the CBT patient (Parra et al., 2013). Furthermore, it has also been proposed that the increased numbers of B cells in CBT recipients is not due the compensatory expansion but due to lymphopoiesis or increased B cell survival induced by B cell activating factor (BAFF), produced by activated monocytes (Batten et al., 2000, Rolink et al., 2002). This current study has shown that CBT recipients have higher absolute numbers of monocytes within peripheral blood and this could explain the higher numbers of B cells within CBT patients. However, further analysis and quantification of BAFF levels in patients would need to be performed to validate this. Although absolute numbers of B cells normalise rapidly after CBT, their functionality has been shown to require significantly longer for recovery. In this current study, functionality of B cells was not addressed, as the B cells were not able to withstand the thawing process for further functionality testing.

There are various studies in HSCT that compare T cell reconstitution in CBT patients compared to BMT and mPB transplant recipients, shown in Table 3.2. However, there is variability in the patients that are investigated including: HLA-

matched and mismatched patients; different conditioning regimens used (myeloablative or reduced intensity); infusion of single or double CB units; various GvHD prophylaxes and patient samples are acquired at differential time points.

Conditioning Regimen		Post-transplant assessment time points (days)	Number of dUCBT patients	Number of comparator patients		Absolute counts of cells post-CBT compared to mPB and BMT		Reference	Absolute counts of cells post-CBT in this current study compared to reference studies	
RIC	MAC			mPB	BMT	CD3+/4+/8+ T cells	CD19+ B cells		CD3+/4+/8+ T cells	CD19+ B cells
-	MAC	45, 90,180 and 365	29	66	-	Lower	Higher	(Kanda et al., 2012)	Lower	Higher
-	MAC	30,60,90,180 and 365	56	59	2	Lower	Higher	(Mehta and Rezvani, 2016)	Lower	Higher
RIC	MAC	90,180 and 365	25	27	9	Lower	Higher	(Servais et al., 2014)	Lower	Higher
RIC	-	30,60,90,180 and 365	241	151	-	Lower	Higher	(Bejanyan et al., 2016)	Lower	Higher
RIC	-	30,60, 90, 180, 365 and 720	42	102	-	Lower	Higher	(Jacobson et al., 2012)	Lower	Higher

Table 3.2 Studies comparing T and B cell absolute counts after CBT compared to different graft sources

In the studies compared in Table 3.2 and from this study, the recurring observation is that there are lower absolute counts of circulating CD3+ and CD4+ T cells in CBT patients compared to healthy controls, mPB and BM transplanted patients. In this current study, it has been shown that there is delayed reconstitution of CD3+ and CD4+ T cells for up to 720 days post-transplant, which validates data from studies listed in Table 3.2. However, in a study by Servais *et al.* it has been shown that there are similar levels of CD4+ T cells compared to mPB recipients, which differs from other studies reporting lower absolute levels of CD4+ T cells post-CBT (Servais et al., 2014).

Aside from CD4+ T cell reconstitution, the absolute counts of CD8+ T cells are also widely reported to reconstitute at 365 days post-transplant, listed in table 3.2. CD8+ T cell reconstitution within this current study shows that these cells reconstitute by 365 days post-CBT. This is in line with other studies shown in Table 3.2.

In CBT patients, T cells can recover via two pathways known as the thymic dependent or independent pathway. The thymic-independent pathway is where mature T cells from the graft expand and proliferate within the recipient. This can be delayed in CBT patients due to the low T cell doses and low numbers of mature T cells within the graft. The thymic dependent pathway occurs where there is production of naïve T cells via progenitor cells that mature within the thymus of the host (Williams et al., 2007). However, there are qualitative and quantitative differences in the CBU graft compared to BM and mPB, which could impact thymic dependent and thymic independent T cell reconstitution in CBT patients. The quantitative difference between CBU and BM and mPB grafts is that there are fewer absolute numbers of HSC and lymphoid progenitor cells in CBU grafts compared to BM and mPB grafts (Szabolcs and Niedzwiecki, 2008). Respectively, the absolute T cell number is also lower in the CB graft, which could cause patients to experience delayed T cell reconstitution post-transplant. This highlights that T cell doses within the graft are an important factor that should be considered during CBU selection as a higher T cell dose could improve T cell reconstitution post-transplant.

T cell reconstitution can also be affected by the intensity of conditioning used pre-transplant. Cytotoxic chemotherapy and radiation negatively impact thymic function and thymic output (Mackall et al., 1995). Furthermore, the administration of GvHD prophylaxis can impact T cell reconstitution (Ogonek et al., 2016). The use of chemotherapeutic agents and GvHD treatment should be further investigated in future CBT studies to assess the level of impact on T cell reconstitution in CBT patients.

ATG has also been shown to impact the reconstitution of CD4+ T cells and patients who are not administered ATG, have a faster reconstitution of these cells post-transplant (Sauter et al., 2011). Patients recruited on to the current study were not administered ATG and still had a delay in CD3+ and CD4+ T cell reconstitution. Furthermore, patients recruited onto the current study had a median age of 48 years old. Thymic involution is age related and is the progressive shrinking of the thymus, which is closely associated with a decline in T cell output from the thymus (Lynch et al., 2009). The patients recruited onto the current study could have reduced thymic output due to thymic involution; this reduces absolute number of T cells released by the thymus and delays T cell reconstitution post -CBT.

CBU are known to contain a higher proportion of naïve T cells that lack antigen-specific memory T cells compared to mPB and BM grafts. These T cells are antigen inexperienced and once they engraft into the patient, they require clonal expansion before inducing a cell-mediated effect. However, as there are a lower number of T cells in the CB graft, the expansion and proliferation of T cells may take longer within the recipient. This is essential within the CBT recipients before T cells can exert a cell-mediated effect to clear pathogenic infections. Therefore, the limited number of antigen specific cells could delay the time in which infections are cleared, making the CBT patient prone to further opportunistic infections (Szabolcs and Niedzwiecki, 2007). To overcome this issue, CBU grafts with a higher T cell number could be used. This could reduce delay in T cell reconstitution. However, a broad TCR repertoire is still required to recognise and clear opportunistic infections whilst balancing an optimised GvL effect.

New approaches are therefore required to improve T cell reconstitution in CBT patients. Factors to improve CD3+, CD4+ and CD8+ T cell reconstitution could include the use of CBU units that are of a higher HLA match. This could be performed by using CBU with a 6/6 HLA match from an unrelated donor and by the use of CMV matched donor before transplantation. These factors have been shown to particularly improve CD4+ T cell reconstitution in paediatric patients (Niehues et al., 2001). Therefore, the use of these approaches could improve the kinetics of T cell reconstitution in adult CBT recipients.

In conclusion, this study shows the reconstitution patterns of the major immune cell subsets in CBT patients. By monitoring the expression patterns of CD45+ as a leucocyte cell marker, we have been able to identify that CD45+ leucocyte cell reconstitution takes place by 60 days post-CBT. Furthermore, CD14+ monocytes reconstitute by 28 days post-CBT. These are two key factors that have not been demonstrated widely in other HSCT and CBT studies.

The current study also validates that the recovery of CD19+ B cells is rapid in CBT patients within the UK, which takes place by 100 days post-CBT (Nakatani et al., 2014). Furthermore, T cells require longer than 1 year to reconstitute in CBT patients. This suggests that there is delayed reconstitution of CD3+, CD4+ and CD8+ T cells post-transplant. Again, this corroborates with data from other CBT studies, detailed in Table 3.2. Herein, it has also been shown that neutrophil engraftment, platelet engraftment and overall survival data is similar to other CBT studies. (Brunstein et al., 2012, Brunstein et al., 2010, Ponce et al., 2011).

However, the initial design of this current study was undertaken to monitor the major immune cell types in CBT patients. This was limited by the flow cytometry acquisition, which only allowed identification of four flourochromes in any single cell analysis. To reduce this limitation, an extensive immunophenotyping panel was devised to investigate the functionality of NK cells in CBT patients, discussed in the next chapter. This allowed for a broader number of flourochromes to be analysed. Furthermore, kinetic studies of reconstitution were set up using genetic techniques to better understand the absolute numbers of B cell and T cell subsets within CBT patients, as discussed in Chapter 5 and 6.

Chapter 4 : NK cells and functionality in cord blood transplant patients

4.1 Background and Aims

NK cells are the first lymphocytes to reconstitute within HSCT recipients irrespective of the graft source. This occurs within the first month of transplant, which has been widely reported in BM, mPB and CBT studies (Brahmi et al., 2001, Buhlmann et al., 2011, Shenoy et al., 1999, Ottinger et al., 1996, Jacobson et al., 2012, Ruggeri et al., 2011, Saliba et al., 2015, Small et al., 1999, Somers et al., 2013, Thomson et al., 2000). NK cell reconstitution is thought to take place as a result of the differentiation of progenitor cells rather than the expansion of mature NK cells within the graft. This theory has been supported by two major observations. Firstly, NK cell reconstitution is not dependent on the type of graft infused into the patient and the NK cell content present within the graft (Abrahamsen et al., 2005, Bjorklund et al., 2010, Martinez et al., 1999). The second major observation is that early NK cells have an immature $CD56^{bright}$ phenotype early post-transplant, which shifts to a $CD56^{dim}$ phenotype after several months post-transplant (Cooley et al., 2005, Nguyen et al., 2008). In healthy adults, up to 90% of NK cells are $CD56^{dim}$ and 10% of NK cells are $CD56^{bright}$. However, the latter subset constitutes most of the NK cells found within the lymph nodes. Within CB, NK cells constitute 15-30% of the mononuclear population. Within the CB NK cell population, 90% are $CD56^{dim}$ and 5-10% are $CD56^{bright}$ (Dalle et al., 2005, Luevano et al., 2012, Kotylo et al., 1990).

NK cells are defined as CD3-CD56+ and can be divided into further subsets depending on their expression of CD56 and CD16. Poli *et al.* has demonstrated that NK cells can be divided into five subpopulations in healthy individuals: (1) $CD56^{bright}$ $CD16^{negative}$; (2) $CD56^{bright}$ $CD16^{positive}$; (3) $CD56^{dim}$ $CD16^{negative}$; (4) $CD56^{dim}$ $CD16^{positive}$ and (5) $CD56^{negative}$ $CD16^{positive}$. The $CD56^{bright}$ $CD16^{negative}$ NK cells comprise 50-70% of the $CD56^{bright}$ NK cell population and the $CD56^{bright}$ $CD16^{dim}$ are 30-50% of the $CD56^{bright}$ NK cell population (Poli et al., 2009).

$CD56^{bright}$ $CD16^{low/negative}$ NK cells are not considered a minor NK cell subpopulation as they can produce a number of different cytokines such as IFN-γ and IL-10 to mediate adaptive immune responses and play an immunoregulatory role (Poli et al., 2009). $CD56^{dim}$ $CD16^{positive}$ NK cells are considered cytotoxic NK cells as they mediate direct lysis of target cells via the release of granules containing perforin and granzyme B, ADCC and activation of cell death pathways such as TRAIL or FAS/FAS-L (Cooper et al., 2001). The role and function of $CD56^{dim}$ $CD16^{negative}$ NK cells is poorly defined and requires further investigation, particularly within CBT patients.

In this current study, there was a focus on the $CD56^{bright}$ $CD16^{negative}$, $CD56^{dim}$ $CD16^{negative}$ and $CD56^{dim}$ $CD16^{positive}$ NK cells to further distinguish their functionality in CBT patients. Much is still unknown about the functions of NK cells post-transplant, particularly how NK cells function in CBT patients. As NK cells are the first lymphocyte to recover, it is likely that they are key mediators and effectors of GvL. Pioneering work by Ruggeri *et al.* showed that NK cells are alloreactive against leukaemic cells after haploidentical transplantation (Ruggeri et al., 2002). Further to this, NK cells with KIR-ligand incompatibility are also able to lyse recipient APCs and this reduces the incidence of GvHD, whilst providing a GvL effect (Ruggeri et al., 2005). With these key effector roles in mind, further studies are required to understand the functions of NK cells post-CBT.

A study conducted by Beziat *et al.* shows that NK cells in CBT patients are functional post-transplant and possess phenotypic features associated with maturity. Furthermore, NK cells in CBT patients can directly lyse leukaemic blast cells, demonstrating a cytotoxic capacity (Beziat et al., 2009). Therefore, it has been proposed that NK cells within CBT patients could be involved in optimised GvL responses and could prevent relapse. Additionally, the cytotoxic capacity of NK cells from CBT patients has been exhibited via direct lysis of leukaemic blast cells such as K562 and RAJI, mediated through ADCC (Beziat et al., 2011). Overall, this displays that NK cells in CBT patients are functionally mature with a cytotoxic capacity to lyse leukaemic cells. Therefore, in this study

the phenotypes of NK cells in CBT patients and the overall capacity of NK cells to lyse leukaemic cells have been investigated.

Additionally, the functions of NK cells are regulated through a number of signals that are delivered through activating and inhibitory receptors. NK cells have a unique property where they are able to eliminate target cells without prior stimulation. However, NK cells can be stimulated by cytokines, which leads to NK cell activation and increased cytolytic activity and proliferation.

CD69 is an early activation marker expressed by activated lymphocytes, including NK cells (Hara et al., 1986). CD69 is involved in functions such as cytokine secretion, proliferation of NK cells and regulation of adhesion molecule expression on NK cells. Additionally, CD69 expressed by NK cells induces cytotoxicity (Borrego et al., 1999, Moretta et al., 1991). HLA-DR is a class II HLA surface receptor, where an initial theory was suggested that it was exclusively expressed by APCs to induce or supress T cell responses (Spits and Lanier, 2007). However, it has been displayed that NK cells express HLA-DR upon activation and that the expression of HLA-DR on NK cells leads to cytokine release to induce CD4+ T cell proliferation (Hanna et al., 2004, Zingoni et al., 2004). Taken together, both CD69 and HLA-DR expression have been investigated in this thesis to understand the activation of NK cells in CBT patients.

NK cell activation can occur through cytokine stimulation and through the recognition of antigens presented on target cells via class I and class II HLA receptors. NKG2C is a C-type lectin activating receptor expressed on NK cells that can recognise HLA-E, a class I molecule expressed on target cells. NKG2C induces signalling through DAP12 signalling molecules and the expression of NKG2C can imply that viral infection is being controlled (Braud et al., 1998, Fang et al., 2011). The increased expression of NKG2C can be associated with viral infections such Hepatitis B and Hepatitis C. Furthermore, the expression of NKG2C is increased in CBT patients infected with CMV, which is a common viral infection post-transplant (Beziat et al., 2012).

NK cells also express a variety of inhibitory receptors that can identify target cells. Normal cells express many of the ligands that are recognised by inhibitory receptors and this is a mechanism that prevents autoimmune killing (Moretta et al., 2001a). NKG2A is an inhibitory receptor expressed by NK cells and like NKG2C can also recognise HLA-E on all nucleated cells. However, their effector function differs compared to NKG2C, where NKG2A receptors transmit an inhibitory signal through the ITIM motif on the cytoplasmic domain. This transduces an inhibitory signal and suppresses the cytolytic activity of NK cells and preventing the lysis of the target cell (Lanier, 2008).

CD57 is an additional marker associated with NK cell activation. CD57 was first thought to be exclusively expressed on NK cells and was used to define NK cell sub-populations. However, it was later revealed that CD57 is also expressed on CD8+ T cells (Abo et al., 1982). CD57 expression was also associated with NK cells that have poor proliferative capacity or even a degree of immunosenescence (Brenchley et al., 2003). More recently, it has been shown that NK cells stimulated by IL-2 have increased CD57 expression, which leads to a mature $CD56^{dim}$ NK cell subpopulation, therefore demonstrating that CD57 is a marker of NK cell maturation (Lopez-Verges et al., 2010). The increased expression of CD57 on NK cells is correlated with infections such as HIV, Hepatitis B and Hepatitis C (Beziat et al., 2012, Hong et al., 2010). Furthermore, as CMV is one of the most common viral infections post-HSCT, it has been shown that these patients have increased expression of CD57 on NK cells compared to non-CMV infected patients (Lopez-Verges et al., 2010).

Taken together, the expression of NKG2A, NKG2C and CD57 are important markers in NK cells in HSCT patients. Early post-transplant, NK cells in HSCT patients express higher levels of inhibitory receptors, NKG2A, compared to healthy controls. During the first three to six months post-transplant, there is loss of the NKG2A expression and increased expression of NKG2C and CD57 (Bjorklund et al., 2010, Dulphy et al., 2008, Nguyen et al., 2005). This suggests that there is a shift towards an activating and functionally mature NK cell phenotype. However, there are discrepancies between studies regarding the reconstitution of NK cell functionality, which can occur anytime between three

and six months post-transplant (Bjorklund et al., 2010, Foley et al., 2011, Vago et al., 2008). With this in mind, the expression patterns of NKG2A, NKG2C and CD57 were measured to determine the kinetics of NK cell maturation in CBT patients.

NK cells are actively involved in inflammatory reactions and participate in immune responses via the production of inflammatory cytokines. IFN-γ is produced by NK cells, which is induced by cytokines such as IL-12 and IL-18 released by macrophages and DCs. Furthermore, TNFα also induces the production of IFN-γ from NK cells (Marshall et al., 2006). The secretion of IFN-γ by NK cells can lead to microbial and tumour clearance and this includes: (1) promotion of the interaction between NK cells and B cells, stimulating isotype switching in B cells to produce antigen specific antibodies; (2) upregulation and expression of adhesion molecules and chemokines and (3) transition between innate immune responses and adaptive immune responses via priming of T helper cells (Hayakawa et al., 2002). Investigating the intracellular expression of IFN-γ within NK cells in CBT patients could allow us to understand whether NK cells in CBT patients produce IFN-γ and whether these NK cells are associated in inflammatory responses.

The cytotoxic capacity of NK cells is important post-transplant, as NK cells could clear residual disease whilst facilitating early engraftment of immune cells following CBT (Clausen et al., 2007, Ruggeri et al., 2002). As previously described, functionally mature CB NK cells can directly lyse leukaemic cell blasts such as K562 and RAJI cells (Tomchuck et al., 2015, Beziat et al., 2009). By understanding the cytotoxic capacity of NK cells in CBT patients, this could allow us to identify whether NK cells are associated with the prevention of early relapse and infection, post-CBT. CBU have a higher percentage of NK cells compared to mPB grafts. However, NK cells within the CB graft have a lower cytotoxic capacity compared to adult PB NK cells (Alnabhan et al., 2015, Luevano et al., 2012). In addition, the cytotoxicity capacity of CB NK cells have been seen to significantly increase upon IL-2 and IL-5 stimulation (Dalle et al., 2005). Overall, this highlights that CB NK cells possess a cytolytic capacity that could be actively involved in GvL responses.

Considering the importance of NK cells in HSCT, the activation of NK cells was investigated in CBT patients. Furthermore, the cytotoxic capacity of NK cells against K562 leukaemic cell blast was analysed.

The specific aims of this chapter are to investigate the following:

1. Identify the percentage expression of NK cell subsets in CBT patients.
2. Identify the expression of activation markers on NK cell subsets within CBT patients.
3. Identify the expression of inflammatory specific NK cells in CBT patients.
4. Identify the percentage expression of IFN-γ on NK cell subsets within CBT patients.
5. Identify the cytotoxic capacity of NK cells in CBT patients.

4.2 Percentage of NK cell subsets in cord blood transplant patients

In this current study, the percentage of NK cell subpopulations in healthy adults and CBT patients were identified. The analysis was performed on isolated and thawed PBMC samples. The PBMCs were processed as described in Chapter 2, section 2.3.2 and 2.3.6, respectively. Three NK cell populations were defined as the following phenotypes: $CD56^{bright}$ $CD16^{dim}$ NK cells, $CD56^{dim}$ $CD16^{positive}$ NK cells and $CD56^{dim}$ $CD16^{negative}$ NK cells. The percentages of the three NK cell populations were calculated as part of the CD56+ NK cell population.

The data in Figure 4.1 shows that at 28 days post-transplant the median percentage of $CD56^{bright}$ $CD16^{dim}$ NK cells (Median: 6%, Interquartile range (IQR) 1-11%) within CD56+ population of patients is equivalent to the percentage seen in PBMCs of healthy adults (7%, IQR: 3-9%). Subsequently, between 60 and 100 days post-CBT the median percentage of $CD56^{bright}$ $CD16^{dim}$ NK cells remained at the same percentage median level compared to healthy controls. Overall, this shows that $CD56^{bright}$ $CD16^{dim}$ NK cells reconstitute within 100 days post-CBT.

The median percentage of $CD56^{dim}$ $CD16^{positive}$ NK cells at 28 days post-CBT (17%, IQR: 14-37%) was significantly lower compared to healthy controls (85%, IQR: 77-89%) ($p=0.0002$). At 60 days post-transplant, the median percentage of $CD56^{dim}$ $CD16^{positive}$ NK cells increased (31%, IQR: 12-44%). However, the median percentage was significantly lower compared to healthy controls ($p=0.002$). The median percentage of $CD56^{dim}$ $CD16^{positive}$ NK cells by 100 days decreased (16%, IQR: 5-38%) compared to day 60. Furthermore, at 100 days post-CBT the median percentage level of $CD56^{dim}$ $CD16^{positive}$ NK cells remained significantly lower compared to healthy controls ($p=0.001$).

At 28 days post-CBT, the median percentage of $CD56^{dim}CD16^{negative}$ NK cells within the CD56+ NK cell population was significantly higher (66%, IQR 48-78%) compared to healthy controls (10%) ($p=0.0002$). Subsequently, at 60 days post-transplant the median percentage of $CD56^{dim}$ $CD16^{negative}$ NK cells remained at a similar level to 28 days and was significantly higher compared to

healthy controls (p=0.002). By 100 days post-CBT, the median percentage of $CD56^{dim}$ $CD16^{negative}$ NK cells within the CD56+ NK cell population increased (77%, IQR: 65-88%) Again, this was significantly higher compared to healthy controls (p=0.001). Overall, this data shows that CBT patients had an equal percentage of $CD56^{bright}$ $CD16^{dim}$ NK cells compared to healthy controls. However, CBT patients had a lower percentage of $CD56^{dim}$ $CD16^{positive}$ NK cells compared to healthy controls. Furthermore, the predominant population of NK cells identified in CBT patients was the $CD56^{dim}$ $CD16^{negative}$ NK cell subset.

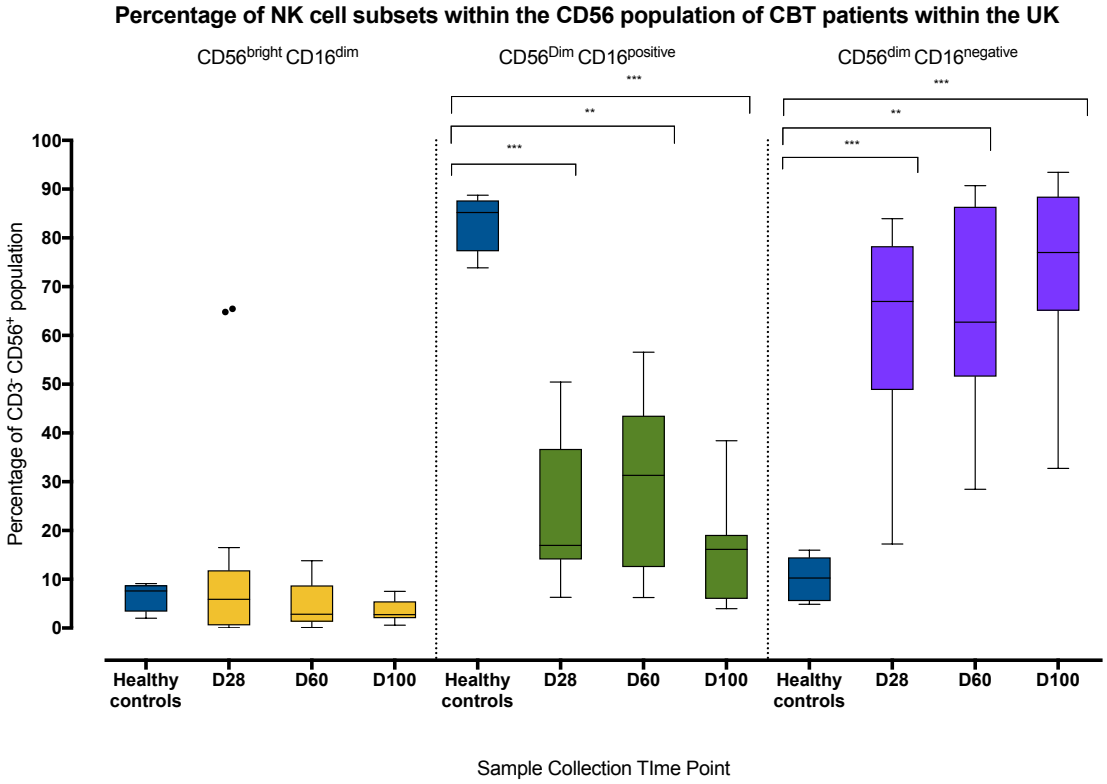


Figure 4.1 Percentage of NK cell subsets in CBT patients. The box and whisker plots represent the percentage of NK cell subsets, quantified within CBT patients. Three subsets were identified within CBT patients: $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cells. At the respective time points the number of patient samples collected were the following: D28: 14, D60: 10 and D100: 10. Furthermore, n=5 healthy control samples were collected. Analysis was performed between patient time points and healthy controls (p= 0.01, p= 0.001). Results are presented as tukey analysis, where a non-paired Mann-Whitney test was performed between sample time points and healthy controls.

4.3 Activation markers of NK cells in cord blood transplant patients

To determine the expression patterns of activation markers in NK cells, thawed isolated PBMCs from healthy adults and CBT patients were used. As previously described, CD69 and HLA-DR are activation markers that are expressed on NK cells that have a cytotoxic function. Both markers have been investigated in this study to better understand whether the NK subpopulations are activated in CBT recipients. The expression of activation markers were measured in $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{positive}$ and $CD56^{dim}$ $CD16^{negative}$ NK cells, as shown in Figure 4.2, 4.3 and 4.4, respectively.

The median percentage of $CD56^{bright}$ $CD16^{dim}$ NK cells expressing CD69 was significantly higher at day +28 (71%), +60 (65%) and +100 (65%) post-CBT compared to healthy controls ($p=0.002$, $p=0.006$ and $p=0.012$, respectively). Furthermore, the median percentage of $CD56^{bright}$ $CD16^{dim}$ NK cells expressing HLA-DR was similar at day +28 (74%), +60 (90%) and +100 (82%) post-CBT compared to healthy controls (79%). Overall, the data shows that the expression of CD69 in the $CD56^{bright}$ $CD16^{dim}$ NK cell population was higher compared to healthy controls. However, the expression HLA-DR was similar in the $CD56^{bright}$ $CD16^{dim}$ NK cell population of CBT patients compared to healthy controls.

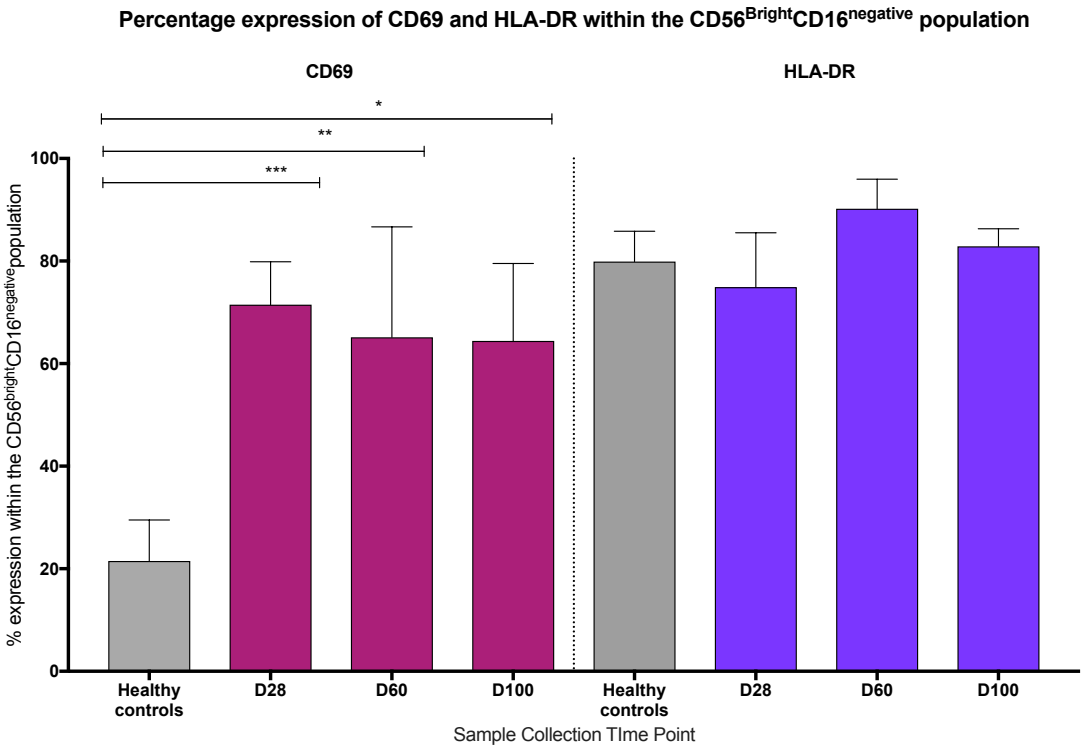


Figure 4.2 Percentage expression of CD69 and HLA-DR in the CD56^{bright} CD16^{negative} NK cell population. The bar chart represents the percentage expression of CD69 and HLA-DR within the CD56^{bright} CD16^{negative} NK cell population of CBT patients. At the respective time points the number of patient samples collected were the following: D28: 14, D60: 10 and D100: 10. Furthermore, n=5 healthy control samples were tested. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls, (p=0.05, p= 0.01, p= 0.001).

The $CD56^{dim}$ $CD16^{positive}$ NK cell population within CBT patients also showed to have a similar pattern of CD69 and HLA-DR expression compared to $CD56^{bright}$ $CD16^{dim}$ NK cells, as seen in Figure 4.3.

The median percentage of $CD56^{dim}$ $CD16^{positive}$ NK cells expressing CD69 was significantly higher at day +28 (84%), +60 (85%) and +100 (83%) post-CBT compared to healthy controls (p=0.0002, p=0.002 and p=0.001, respectively). Furthermore, the median percentage of $CD56^{dim}$ $CD16^{positive}$ NK cells expressing HLA-DR was similar at day +28 (72%) compared to healthy controls (72%). However, the median percentage of $CD56^{dim}$ $CD16^{positive}$ NK cells expressing HLA-DR was significantly higher at day +60 (82%) and +100 (85%) post-CBT compared to healthy controls (72%) (p=0.002 and p=0.007, respectively). Overall, this demonstrates that the percentage expression of CD69 is higher in $CD56^{dim}CD16^{positive}$ NK cells of CBT patients compared to healthy controls. However, HLA-DR is similarly expressed in $CD56^{dim}CD16^{positive}$ NK cells of CBT patients compared to healthy controls.

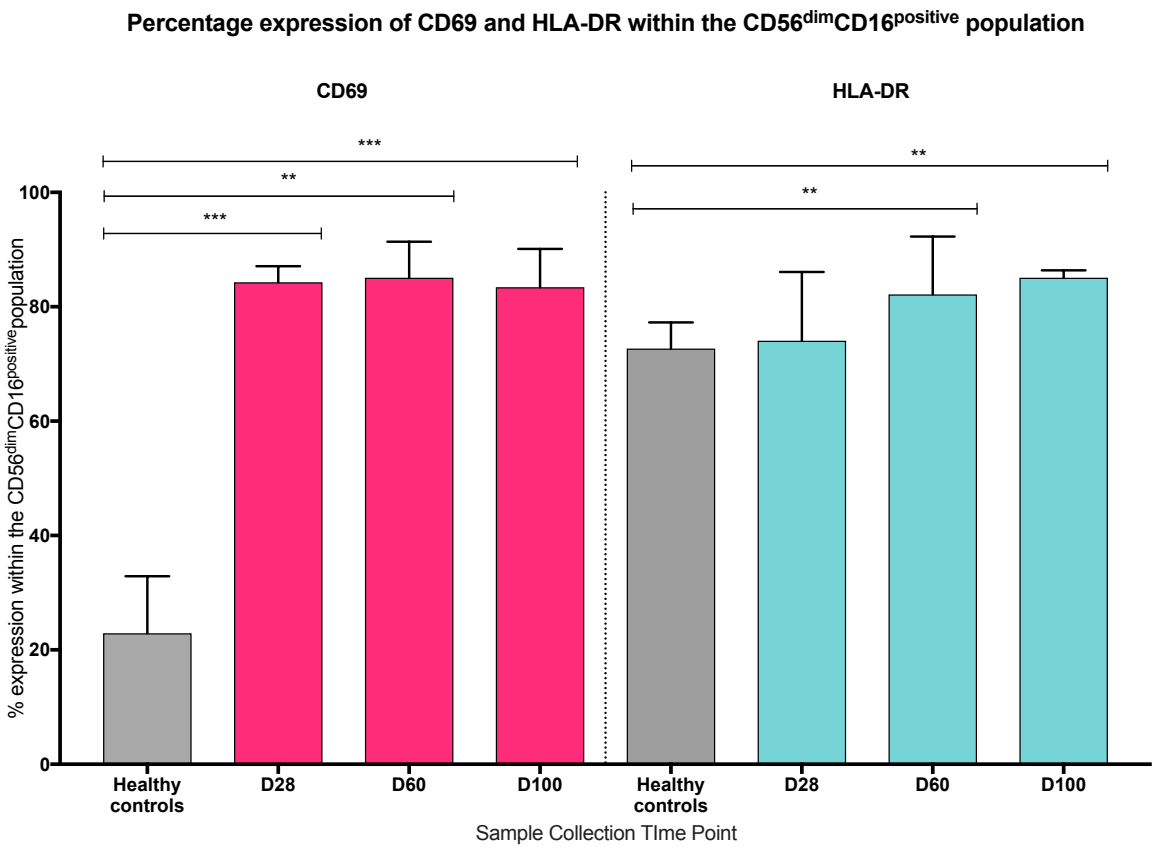


Figure 4.3 Percentage expression of CD69 and HLA-DR in the CD56^{dim} CD16 ^{positive} NK cell population. The bar chart represents the percentage expression of CD69 and HLA-DR within CD56^{dim} CD16^{positive} NK cells in CBT patients. At the respective time points the number of patient samples collected were the following: D28: 14, D60: 10 and D100: 10. Furthermore, n=5 healthy control samples were tested. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls, (p= 0.01, p= 0.001).

The pattern of CD69 and HLA-DR expression was also similar in $CD56^{dim}$ $CD16^{negative}$ NK cells compared to $CD56^{bright}$ $CD16^{dim}$ and $CD56^{dim}$ $CD16^{positive}$ NK cells, as seen in Figure 4.4.

The median percentage of $CD56^{dim}$ $CD16^{negative}$ NK cells expressing CD69 was significantly higher at day +28 (84%), +60 (83%) and +100 (86%) compared to healthy controls (43%) ($p=0.0002$, $p=0.002$ and $p=0.001$, respectively). The median percentage of $CD56^{dim}$ $CD16^{negative}$ NK cells expressing HLA-DR was higher at day +28 (73%). Additionally, the median percentage of $CD56^{dim}$ $CD16^{negative}$ NK cells expressing HLA-DR was significantly higher day +60 (84%) and +100 (92%) compared to healthy controls (64%) ($p=0.002$ and $p=0.001$, respectively). Overall, this data suggests that the median percentage expression of CD69 and HLA-DR is higher in CBT patients compared to healthy controls and remains higher compared to healthy controls for up to 100 days post-transplant.

Taken together, our data shows that CBT patients have activated NK cells post-transplant and this is shown via the expression of NK cell markers such as CD69 and HLA-DR. As NK cells in CBT patients were activated, the expression patterns of activating and inhibitory (NKG2A, NKG2C and CD57) receptors were also measured post-transplant.

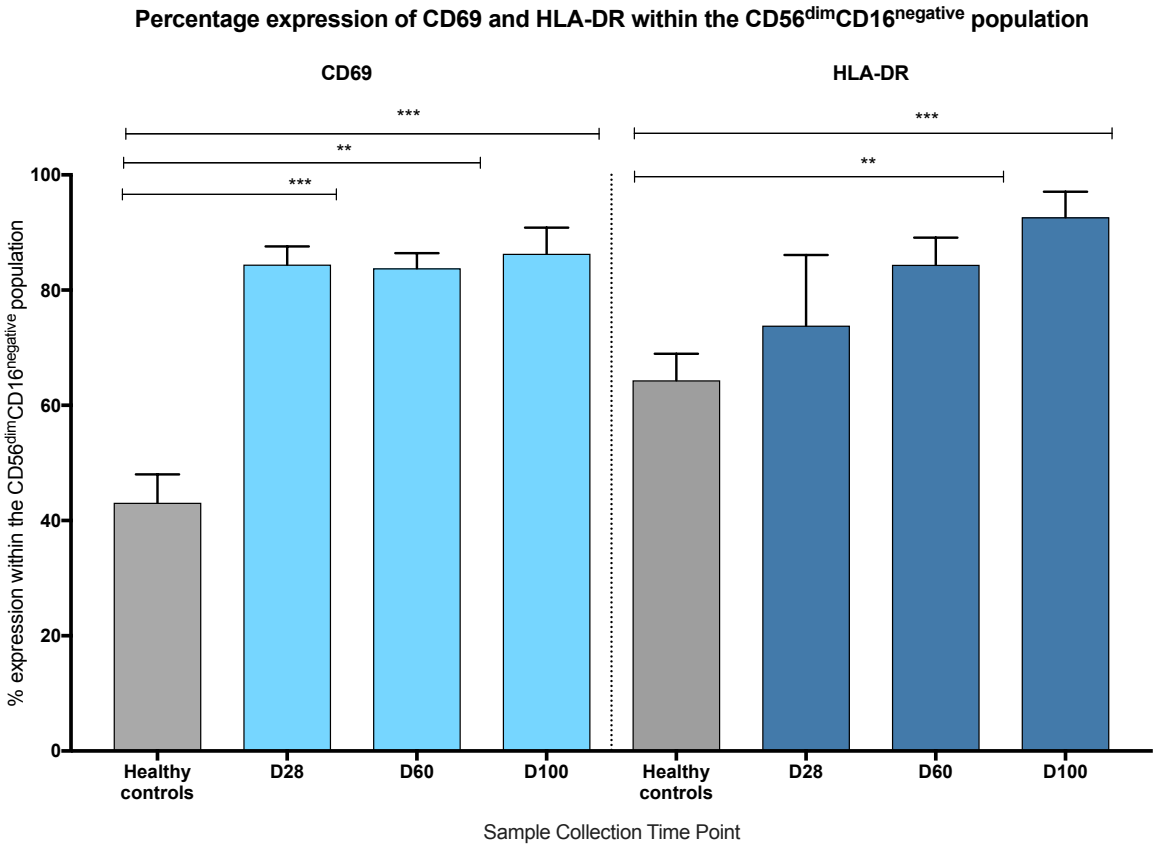


Figure 4.4 Percentage expression of CD69 and HLA-DR in the CD56^{dim} CD16^{negative} NK cell population. The bar chart represents the percentage expression of CD69 and HLA-DR within CD56^{dim} CD16^{negative} NK cells in CBT patients. At the respective time points the number of patient samples collected were the following: D28: 14, D60: 10 and D100: 10. Furthermore, n=5 healthy control samples were tested. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls (p= 0.01, p= 0.001).

4.4 Activating and inhibitory receptors of NK cells in cord blood transplant patients

The pattern of activating and inhibitory marker expression of NKG2A, NKG2C and CD57 were identified in CBT patients for up to 100 days post-transplant as shown in Figure 4.5, 4.6 and 4.7. To determine the expression patterns of activating and inhibitory receptors in NK cells, thawed isolated PBMCs from healthy adults and patients were used.

The percentage expression of NKG2A and NKG2C was measured in NK cells of CBT patients to understand the patterns of the respective activation and inhibitory receptors. As previously described, CMV is a common viral infection post-transplant and patients have increased expression of NKG2C on NK cells post-infection. Increased expression of NKG2C on NK cells can lead to direct lysis and cytotoxicity of the target cell. Alternatively, NKG2A is an inhibitory receptor that suppresses the cytolytic activity of NK cells. CD57 coupled with NKG2A or NKG2C demonstrates NK cell maturation.

The median percentage of $CD56^{bright}CD16^{dim}$ NK cells expressing NKG2A+CD57+ was significantly higher at day +28 (5%), +60 (9%) post-CBT compared to healthy controls (0%) ($p=0.03$ and $p=0.02$, respectively). Furthermore, the median percentage of $CD56^{bright}$ $CD16^{dim}$ NK cells expressing NKG2A+CD57+ was higher at day +100 (3%) post-CBT compared to healthy controls (0%). There was no pattern of expression of NKG2C+CD57+ in $CD56^{bright}$ $CD16^{dim}$ NK cells and expression levels remained below 1% up to 100 days post-CBT. This is lower compared to healthy controls, where a median expression was recorded at 20%. This demonstrates that the percentage expression of NKG2A+CD57+ within $CD56^{bright}$ $CD16^{dim}$ NK cells population is higher compared to healthy controls and the level of expression of NKG2C+CD57+ within $CD56^{bright}$ $CD16^{dim}$ NK cells is lower compared to healthy controls. Therefore, this indicates that $CD56^{bright}$ $CD16^{dim}$ NK cells have a higher expression of inhibitory receptors compared to healthy controls. This could indicate that $CD56^{bright}$ $CD16^{dim}$ NK cells are mature with an inhibitory phenotype.

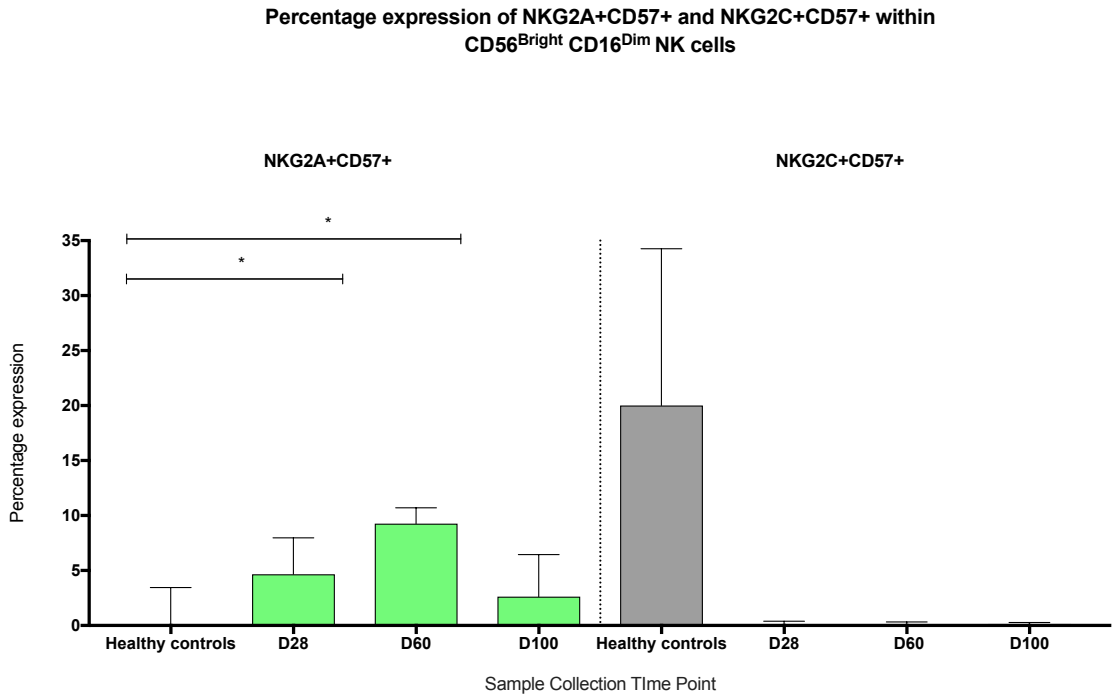


Figure 4.5 Percentage expression of NKG2A+CD57+ and NKG2C+CD57+ expression within the CD56^{bright} CD16^{dim} NK cell population. The bar chart represents the percentage expression of NKG2A+CD57+ and NKG2C+ and CD57+ expression within the CD56^{bright} CD16^{dim} NK cell population in CBT patients. At the respective time points the number of patient samples collected were the following: D28: 14, D60: 10 and D100: 10. Furthermore, n=5 healthy control samples were tested. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls (p= 0.05).

The median percentage of $CD56^{dim}CD16^{positive}$ NK cells expressing NKG2A+CD57+ was significantly higher at day +28 (7%), +60 (14%) +100 (15%) post-CBT compared to healthy controls (3%) (p=0.02, p=0.007 and p=0.02, respectively). Throughout the three time points, the expression of NKG2C+CD57+ was not detected on $CD56^{dim}$ $CD16^{positive}$ NK cells. Overall, this data suggests that the $CD56^{dim}$ $CD16^{positive}$ NK cell population also have an inhibitory phenotype due to the increased expression of NKG2A+CD57+.

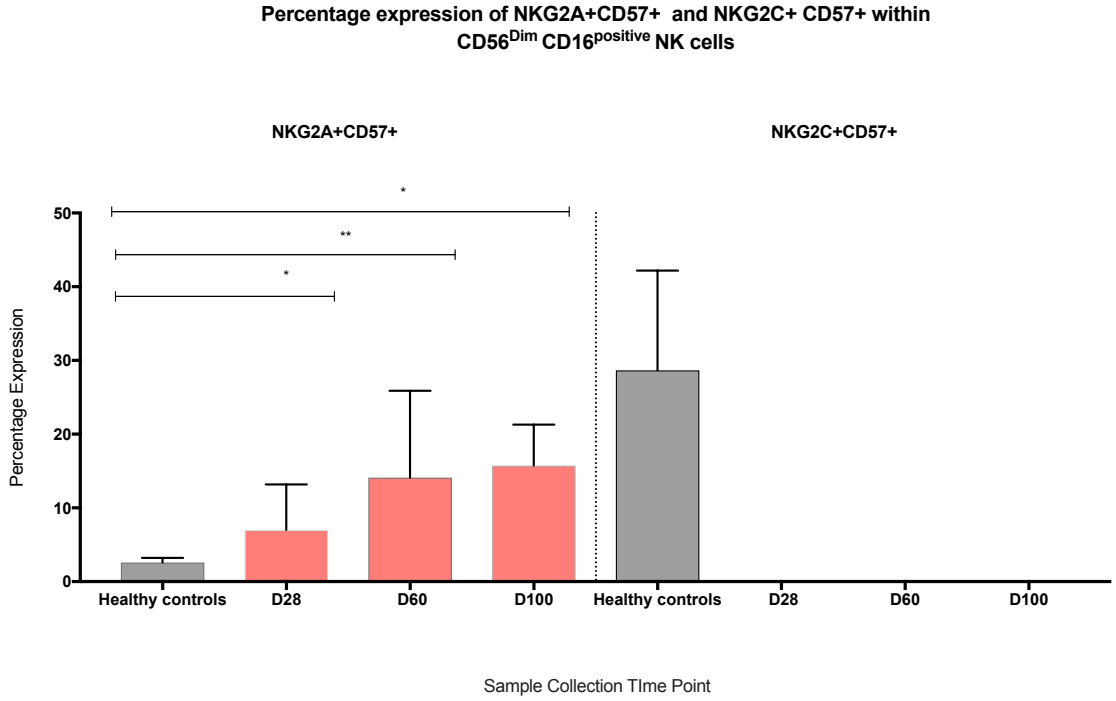


Figure 4.6 Percentage expression of NKG2A+CD57+ and NKG2C+CD57+ within the $CD56^{dim}$ $CD16^{positive}$ NK cell population. The bar chart represents the percentage expression of NKG2A+CD57+ and NKG2C+CD57+ within the $CD56^{dim}$ $CD16^{positive}$ NK cell population in CBT.. At the respective time points the number of patient samples collected were the following: D28: 14, D60: 10 and D100: 10. Furthermore, n=5 healthy control samples were tested. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls, (p= 0.05 and p=0.01).

The median percentage of CD56^{dim} CD16^{negative} NK cells expressing NKG2A+CD57+ was significantly higher at day +28 (4%) and +60 (7%) post CBT compared to healthy controls (p=0.04 and p=0.01, respectively). Furthermore, the median percentage of CD56^{dim} CD16^{negative} NK cells expressing NKG2A+CD57+ was higher at day +100 (15%) post-CBT compared to healthy controls (3%). The expression of NKG2C+CD57+ was undetected within the CD56^{dim} CD16^{negative} NK cell population of CBT patients. Overall, this data shows that CD56^{dim} CD16^{negative} NK cells could have an inhibitory phenotype as expression levels of NKG2A+CD57+ are higher compared to healthy controls. Furthermore, there is no expression of NKG2C+CD57+ on CD56^{dim} CD16^{negative} NK cells within CBT patients suggesting that there is no activation of this cell subset post-transplant.

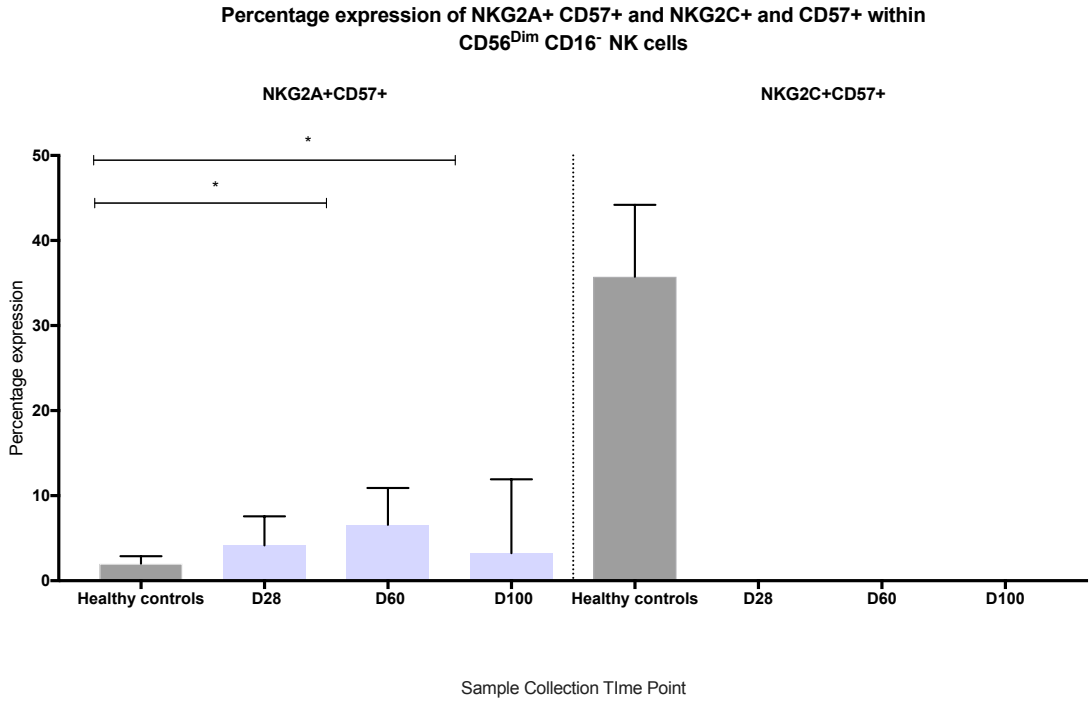


Figure 4.7 Percentage expression of NKG2A+CD57+ and NKG2C+CD57+ within the CD56^{dim} CD16^{negative} NK cell population. The bar chart represents the percentage expression of NKG2A+CD57+ and NKG2C+CD57+ expression within the CD56^{dim} CD16^{negative} NK cell population in CBT patients. At the respective time points the number of patient samples collected were the following: D28: 14, D60: 10 and D100: 10. Furthermore, n=5 healthy control samples were tested. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls (*p*= 0.05).

4.5 Percentage expression of intracellular IFN-γ within the CD56^{bright} CD16^{negative}, CD56^{dim} CD16^{positive} and CD56^{dim} CD16^{negative} NK cells in cord blood transplant patients.

IFN-γ is a pro-inflammatory cytokine released by NK cells. The release of IFN-γ from NK cells mediates clearance of microbes and tumours through a number of pathways, as previously described. Understanding the pattern of IFN-γ expression in CBT patients could demonstrate that NK cells can produce IFN-γ but also that these NK cells could be involved in GvL responses and clearing infection post-transplant. Additionally, identifying the intracellular expression of IFN-γ in NK cells within CBT patients could imply that CBT patients have NK cells with a cytotoxic capacity. The patterns of IFN-γ intracellular expression are shown in Figure 4.8a, 4.8b and 4.8c. The expression of IFN-γ has been measured in non-stimulated conditions. Thawed isolated PBMCs from healthy adults and patients were used to determine the intracellular expression patterns IFN-γ in NK cells.

The median percentage of CD56^{bright} CD16^{negative} NK cells expressing IFN-γ (Figure 4.8a) was higher at day +28 (70%) and +60 (80%) and +100 (64%) post-CBT compared to healthy controls (0%). Additionally, the median percentage of CD56^{dim} CD16^{positive} NK cells expressing IFN-γ (Figure 4.8b) was significantly higher at day +28 (66%) and +60 (67%) and +100 (62%) post-CBT compared to healthy controls (0%) ($p=0.0001$, $p=0.0007$ and $p=0.0007$). The median percentage of CD56^{dim} CD16^{negative} NK cells expressing IFN-γ (Figure 4.8c) was significantly lower at day +28 (64%) and +60 (68%) and +100 (62%) post-CBT compared to healthy controls (77%) ($p=0.03$ and $p=0.02$). Overall, this data demonstrates that all three NK cell populations in CBT patients express IFN-γ intracellularly and the expression is highest within the CD56^{bright} CD16^{negative} and CD56^{dim} CD16^{positive} NK cell populations. However, within the CD56^{dim} CD16^{negative} NK cell population intracellular expression of IFN-γ is similar to healthy adults.

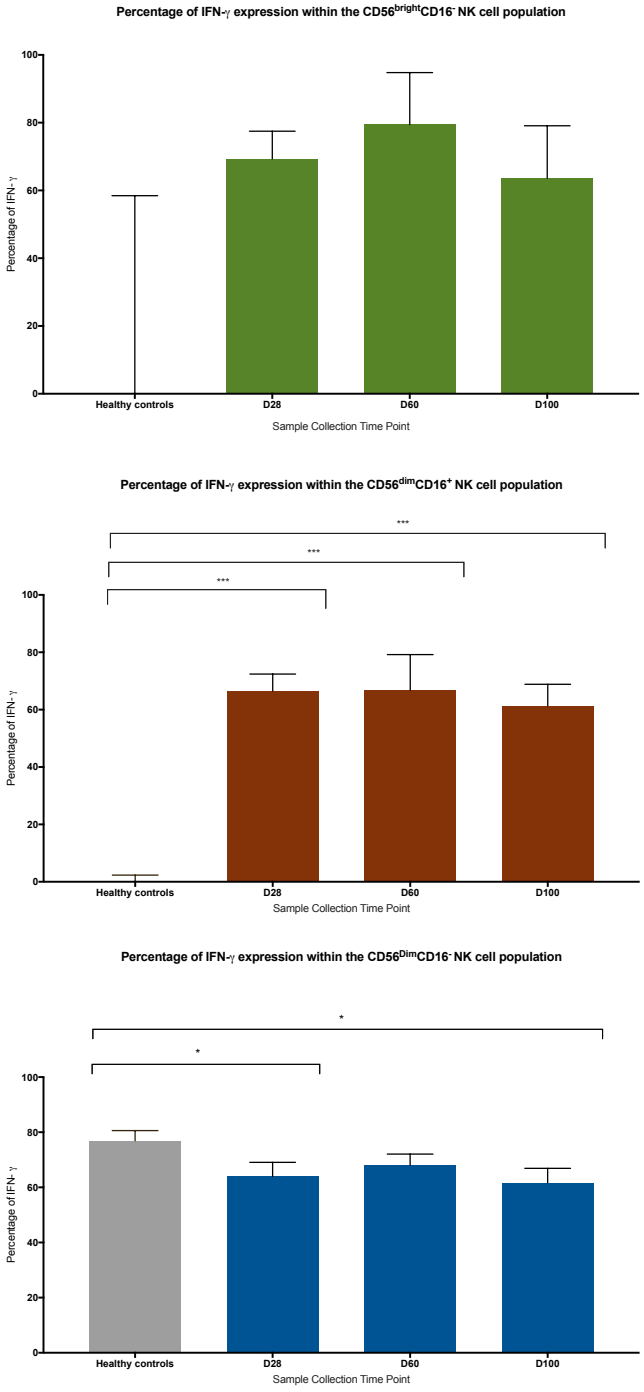


Figure 4.8 Intracellular expression of IFN-ɣ in CD56^{bright} CD16^{negative}, CD56^{dim}CD16^{positive} and CD56^{dim}CD16^{negative} NK cells. The bar charts represent the percentage expression of IFN-ɣ within (A) CD56^{bright}CD16^{negative}, (B) CD56^{dim}CD16^{positive} and (C) CD56^{dim}CD16^{negative} NK cells in CBT patients. At the respective time points the number of patient samples collected were the following: D28: 14, D60: 10 and D100: 10. Furthermore, n=5 healthy control samples were tested. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls. (p= 0.05 and p= 0.001).

4.6 Killing capacity of cord blood transplant NK cells

In this current study, the cytotoxic capacity of isolated NK cells from CBT patients was analysed. To determine the cytotoxic capacity, NK cells were isolated from thawed PBMCs and were then tested in an *in vitro* killing assay. Our previous data shows that NK cells are activated via the expression of CD69 and HLA-DR. Respectively, NK cells have a higher expression of the inhibitory marker NKG2A. Furthermore, all of the NK cell subsets express IFN-ɣ intracellularly, which could promote the cytotoxic activity of NK cells. Based on these findings it was hypothesised that NK cells in CBT patients are cytotoxic. This hypothesis was tested by measuring the cytotoxicity capacity of NK cells directed towards leukaemic cells (K562) at a 1:1 ratio as described in Chapter 2, Section 2.8.8. Thawed isolated NK cells from PBMCs of patients and healthy adult control samples were incubated with K562 cells for four hours and the percentage reduction of the K562 population was measured via flow cytometry.

The data in Figure 4.9 shows that NK cells had a cytotoxic capacity to K562 leukaemic cell blasts at all time points post-CBT. The median percentage of specific cell lysis of K562 cells is shown at the following time points post-transplant: day 28, 60, 100 and 180.

The median percentage of specific K562 cell lysis at day +28 (36%, range: 19.48-95.15%), +60 (44%, range: 31.72–71.85%), +100 (18%, range: 5.43–73.84%) and +180 (39%, range: 5.87-79.96%) post-CBT, shows that CBT patient NK cells are capable of lysing K562 leukaemic cell blasts to the same degree as healthy controls (32%, range: 6.27 – 78.2%). Furthermore, patient NK cells are able to direct killing towards K562 cells at a 1:1 ratio.

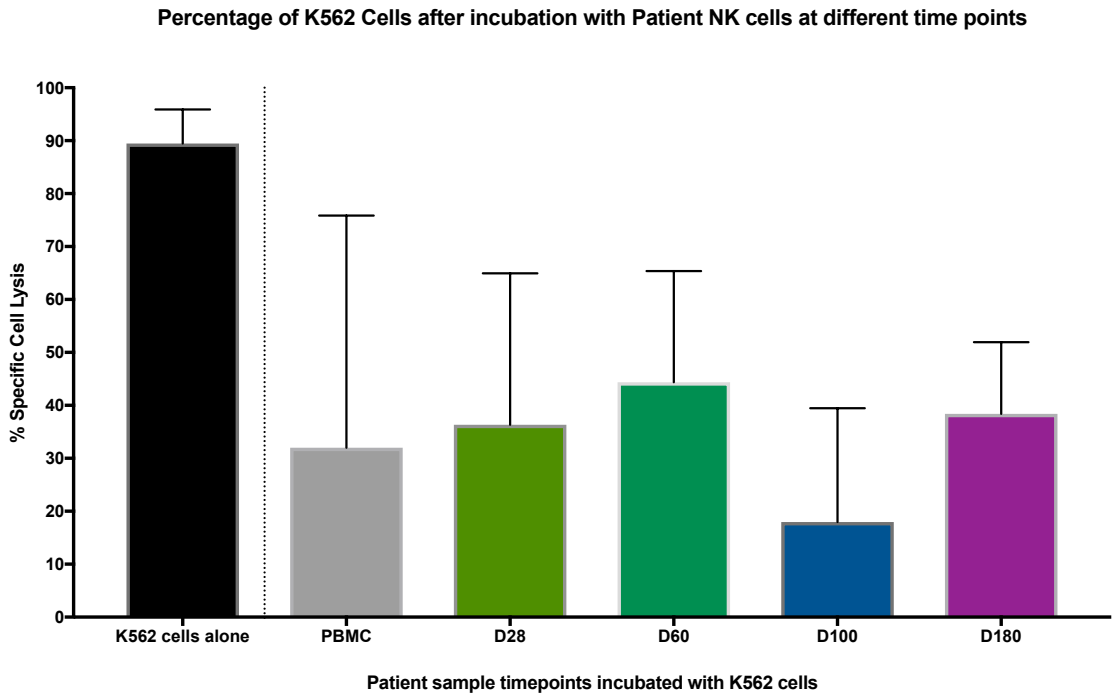


Figure 4.9 Killing of K562 cells *in vitro* by patient NK cells and healthy control NK cells. The bar chart represents the percentage of K562 cells lysed by patient NK cells. Isolated CD56+ NK cells from patient samples were incubated with K562 cells for four hours and the percentage of K562 cells were measured via flow cytometry. At the respective time points the number of patient samples collected were the following: D28: 14, D60: 10, D100: 10 and D180: 10. Furthermore, n=5 healthy control samples (PBMCs) were tested for this assay. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls.

4.7 Discussion

As far as we are aware, this is the first study within the UK to investigate the post-transplant functionality of NK cells in CBT patients. Various studies have assessed the phenotype of CB NK cells and PB NK cells. However, there is limited data on the functionality of NK cells in a post-CBT setting.

NK cells are the first lymphocyte population that reconstitute in CBT patients and this occurs within one month post-transplant (Jacobson et al., 2012, Ruggeri et al., 2011). T cell reconstitution is delayed compared to NK cells. However, as NK cells reconstitute before T cells they are believed to have a GvL role post-transplant (Ruggeri et al., 2002). This has not been thoroughly investigated in CBT patients and the phenotype and cytotoxic activity of NK cells was assessed in this study to better understand the function of these cells post-transplant.

In this study, it is shown that there are three NK cell subpopulations within CBT patients: $CD56^{bright}$ $CD16^{negative}$, $CD56^{dim}$ $CD16^{negative}$ and $CD56^{dim}$ $CD16^{positive}$ NK cells. There were a higher percentage of $CD56^{dim}$ $CD16^{negative}$ NK cells in CBT patients compared to healthy controls and a lower percentage of $CD56^{dim}$ $CD16^{positive}$ NK cells compared to healthy controls. However, little is known about $CD56^{dim}$ $CD16^{negative}$ NK cells and future NK cell studies should be undertaken to identify the functionality of these cells in HSCT, particularly within CBT patients. As previously described, Poli *et al.* considered $CD56^{dim}$ $CD16^{negative}$ NK cells to be one of the sub-populations of NK cells and this group has also stated that little is known about the function of this subset (Poli et al., 2009) In addition, a study conducted by Romee *et al.* highlights that NK cells down regulate CD16 expression and $CD56^{dim}$ $CD16^{positive}$ NK cells shed and down regulate CD16 upon exposure to tumour targets (Romee et al., 2013). Furthermore, $CD56^{dim}$ $CD16^{positive}$ NK cells lose CD16 expression through the action of matrix metalloproteinase enzymes upon NK cell interaction with tumour antigens (Grzywacz et al., 2007).

$CD56^{dim}$ NK cells have cytolytic effector functions and can lyse target cells. Additionally, $CD56^{dim}$ NK cells are a major source of pro-inflammatory cytokines and chemokines, which are rapidly produced after target cell recognition. Furthermore, $CD56^{dim}$ NK cells can exert a higher cytotoxic function compared to $CD56^{bright}$ NK cells (Cooper et al., 2001).

Complete phenotypic and functional maturation of NK cells can take up to several months post-transplant. Early post-transplant, patients have a high proportion of immature $CD56^{bright}$ NK cells. This shifts to a high proportion of $CD56^{dim}$ NK cells within several months (Nguyen et al., 2008). The analysis in this current study was performed on patient samples within the first 100 days post-CBT. The data shows there were a high proportion of $CD56^{dim}$ NK cells, early post-transplant. This corroborates data by Nyugen *et* al (Nguyen et al., 2008). Therefore, the phenotypic shift from $CD56^{bright}$ to a $CD56^{dim}$ NK cell population could demonstrate that there is reconstitution of $CD56^{dim}$ $CD16^{negative}$ and $CD56^{dim}$ $CD16^{positive}$ NK cell subsets. However, to further understand the phenotype and function of these NK cell subsets, further studies are required to elucidate their role.

Data within this current study shows that the expression of CD69 is higher in CBT patients early post-transplant compared to healthy controls. Furthermore, the expression levels of CD69 are significantly higher in all three NK cell populations compared to healthy controls. This corroborates data presented by Beziat *et al.* who has shown that NK cell subsets have increased expression of CD69, early post-transplant and this indicates early activation of NK cells in CBT patients (Beziat et al., 2009). In addition, this current study also shows that the NK cell subpopulations in CBT patients had a higher expression of HLA-DR compared to healthy controls. This also highlights that NK cells are activated post-CBT. When combined, high expression of CD69 and HLA-DR in CBT patients compared to healthy controls shows that NK cells are activated post-transplant.

CBT-patients can be challenged by a number of infections post-transplant and the various pathogenic antigens could lead to NK cell activation. Higher

expression of HLA-DR by NK cells is associated with the release of pro-inflammatory cytokines, suggesting that a functional immune response takes place to lyse the target cell (Zingoni et al., 2004). Overall, this suggests that NK cells within CBT patients are activated post-transplant.

Herein, it has been shown that NK cells in CBT patients are activated. The expression patterns of NKG2A+CD57+ and NKG2C+CD57+ were further investigated to understand the expression patterns of activating and inhibitory receptors in CBT patients. Through this analysis, it has been shown that there is a very low level of NKG2C+CD57+ expression within CBT patients compared to healthy controls. However, CBT patients have a higher expression of NKG2A+CD57+ compared to healthy controls in all three NK cell subpopulations. This could suggest that NK cells, early post-transplant, have an inhibitory phenotype in CBT patients. A study conducted by Kordelas *et al*. demonstrates that patients with severe GvHD have significantly reduced levels of NKG2C after allogeneic HSCT (Kordelas et al., 2016). However, this could not be correlated in this current study, as GvHD data was not complete. The expression patterns of NKG2A+CD57+ or NKG2C+CD57+ could therefore be used to distinguish whether there are mature-activating or mature-inhibitory NK cells post-CBT, respectively. In a study conducted by Romee *et al.* it has been observed that NK cells have a mature-inhibitory phenotype, early post-transplant, with increased expression of NKG2A. Furthermore, the expression of NKG2A is induced by IL-12 and IL-18 (Romee et al., 2012). This could suggest that NK cells, early post-CBT, have a memory like phenotype and their phenotype is induced via cytokine stimulation. However, further studies would be required to assess the presence and level of cytokines in CBT patients and whether patient NK cells can be stimulated via cytokines.

Additionally, the intracellular expression of IFN-γ was higher in the $CD56^{bright}$ $CD16^{negative}$ and $CD56^{dim}CD16^{positive}$ NK cell populations compared to healthy controls. However, the intracellular expression of IFN-γ was equal in the $CD56^{dim}CD16^{negative}$ NK cell subpopulation of CBT patients compared to healthy controls. The intracellular expression of IFN-γ in all three NK cell populations could be due to the activation of NK cells within the patient. Post-transplant, it is

known that the immune profile of a patient is highly inflammatory and this is due to the presence of inflammatory cytokines such as IL-1, IL-6 and TNF-α. Additionally, the immune system will be challenged by a number of foreign antigens. In turn, this could induce the activation of NK cells, which could subsequently cause the intracellular production of IFN-γ within NK cells of CBT patients. IFN-γ is released by NK cells and mediates clearance of microbes and tumours through a number of pathways, as previously described. Data within this current study corroborates published data by showing that IFN-γ expression is higher in CBT patients compared to healthy controls (Beziat et al., 2009). CBT patients are immunocompromised post-transplant and are administered a number of immunosuppressive agents as part of their GvHD prophylaxis. Early post-transplant, the use of immunosuppressive agents can suppress cytokine release and the levels of IFN-γ can be impaired, however the expression IFN-γ normalises after several months (Melenhorst et al., 2012). Furthermore, CMV infection has been shown to influence IFN-γ expression on NK cells and CMV infection induces rapid maturation of NK cells with the increased expansion of $CD56^{dim}$ NK cells. (Della Chiesa et al., 2014). Overall, there was a higher proportion of $CD56^{dim}$ NK cells in CBT patients within this current study and these cells were capable of expressing IFN-γ. However, in this current study, infection data was not complete and therefore correlations of infection with $CD56^{dim}$ NK cell percentages could not be made. Therefore, further studies must be undertaken to understand whether CMV infection impacts NK cell maturation post-CBT.

The cytotoxic functions of NK cells are highly important and there is limited of data that defines the cytotoxic capacity of NK cells in CBT patients. Herein, NK cells from CBT patients can mount a cytotoxic response to K562 cells, which verifies data demonstrated by Beziat *el al.* who performed this analysis on freshly isolated NK cells (Beziat et al., 2009). However, in this current study, to understand the cytotoxic capacity of NK cells, thawed and isolated NK cells were used. These NK cells can also mount a cytotoxic response against K562 cells. Unfortunately, cytolytic degranulation of NK cells could not be identified as the cell viability of NK cells reduced upon staining of CD107a. To understand the cytotoxic capacity of NK cells in CBT patients, further work can be done by

conducting *in vitro* killing assays on alternative cell lines such as 721.221 and Jurkat cells. Furthermore, the use of patient AML cells could also provide a better understanding of NK cell cytotoxic capacity. This could enhance our understanding of the cytotoxic capacity of NK cells and whether they can lyse primary malignant cells.

To summarise, the predominant NK cell subpopulation in CBT patients was $CD56^{dim}$ $CD16^{negative}$. All three NK cell sub-populations were activated with high expression of CD69 and HLA-DR. NK cell subpopulations in CBT patients had an inhibitory phenotype and all sub-populations expressed IFN-γ intracellularly. The cytotoxic capacity of NK cells was measured and NK cells from CBT recipients can directly lyse K562 leukaemic blast cells. Herein, NK cells in CBT patients showed a functional capacity.

Chapter 5 : Kinetics and diversity of reconstituting B cell subsets in cord blood transplant patients.

5.1 Background and Aims

In Chapter 3, it was shown that CD19+ B cell reconstitution occurs by 100 days post-transplant. In contrast, patterns of B cell reconstitution have been investigated in several mPB and BMT studies, which have shown that absolute B cell reconstitution takes place between 180 and 365 days post-transplant (Storek et al., 1993, Storek et al., 2001b, Avanzini et al., 2005, Lev et al., 2012). Comparatively, CBT recipients have a faster B cell reconstitution, which takes place between 100 and 180 days post-CBT (Nakatani et al., 2014).

B cells play a crucial role in humoral immune responses and a diverse B cell repertoire is key for an effective immune response. Healthy adult individuals have a broad B cell repertoire enabling them to recognise a variety of self and non-self antigens. In HSCT, it has been demonstrated that reconstituting B cells could have a diverse BCR repertoire, as early as 90 days post-transplant (Beaudette-Zlatanova et al., 2013). This highlights that early B cell reconstitution and increased BCR diversity is required as part of reconstitution of adaptive immunity. This is essential as CBT patients are prone to a number of opportunistic infections within the first few months post-transplant. In addition, if complete B cell reconstitution takes place early post-transplant, then immune defence mechanisms can be primed to clear opportunistic infections. Furthermore, in CBT patients, early B cell reconstitution is associated with lower incidence of GvHD. This could be due to a higher absolute number of B cells that reconstitute earlier post-transplant compared to BMT and mPB transplant recipients (Jacobson et al., 2012).

With these important functions of B cells in mind, the diversity and kinetics of B cell reconstitution was measured in CBT patients. Overall, this was measured to

determine the time in which B cell recovery takes place and to understand whether the sequential maturation stages take place during B cell reconstitution.

CB grafts predominantly contain naïve B cells, which could mean that there are a high proportion of B cells with low BCR diversity and these cells have not been challenged by antigens. Initial B cell development takes place in fetal tissue and in the BM. Once HSCs differentiate into CD19+ pro-B cells, gene rearrangement of the immunoglobulin heavy chain and the Ig light chain locus takes place. This allows maturation of a pro-B cell into a pre-B cell. Subsequently, further gene arrangement takes place within a pre-B cell and the light chain of the Ig locus rearranges to give rise to a functional immature B cell (Cooper, 1987, Uckun, 1990). During Ig locus gene recombination in B cells there is production of excision circles known as kappa deleting recombination circles (KRECs). KRECs are formed during B cell maturation within the BM and are bi-products of recombination events that cause exclusion of the Ig kappa locus (Siminovitch et al., 1985, van Zelm et al., 2007). This takes place within B cells that have failed to reproductively rearrange the Ig kappa gene on both alleles (Beishuizen et al., 1997). The Ig kappa gene becomes non-functional and this leads to deletion of the gene segment. Furthermore, this leads to the formation of a coding joint and excision loop (KRECs), which is excluded from further gene rearrangements (Beishuizen et al., 1997).

Naïve/immature and transitional B cells released into the periphery from the BM contain high levels of KRECs and as these B cells proliferate and mature the copy number of KRECs dilute. The measurement of KRECs provides an indication of B cell output from the BM. KREC copy numbers increase in allogeneic HSCT patients for up to 180 days post-transplant and this correlates with increased absolute numbers of naïve, transitional/immature B cells (Mensen et al., 2013). In CBT recipients, there is rapid neogenesis of B cells compared to BMT recipients, which has been confirmed through KRECs quantification (Nakatani et al., 2014). Therefore, this highlights that KREC quantification can be used as an important genetic marker to monitor BM output

of B cells. With this in mind, KREC quantification was performed in this current study to better understand the output of B cells from the BM in CBT patients.
B cell maturation within the BM is a highly important stage in B cell development. However, there are a number of developmental stages involved in B cells maturation, which occur within the periphery. These are essential stages that are required for the production of antibody secreting B cells, which are one of the most mature B cell populations. In this project, the kinetics and recovery of B cell subsets at the various maturation stages in CBT patients have been identified and quantified.

Once B cells mature within the BM they differentiate into transitional B cells and are released into the peripheral blood system. Transitional B cells express cell surface markers such as CD24 and CD38. Through the use of these cell surface markers, transitional B cells can be characterised as CD4-CD19+CD24+CD38+. A study conducted by Marie-Cardine *et al.* shows that transitional B cells are the first B cell subpopulation to reconstitute after HSCT. Furthermore, they are defined as an important developmental stage, which is required before differentiation into mature B cells (Marie-Cardine et al., 2008). As far as we are aware, the kinetics of transitional B cell reconstitution has not been formally investigated in CBT patients. Therefore, defining the kinetics of transitional B cell reconstitution will demonstrate whether this key developmental stage takes place post-transplant.

Once transitional B cells develop they migrate from the BM to the spleen and differentiate into naïve B cells. In the spleen, naïve B cells are presented with a number of antigens, which results in maturation and differentiation into antibody class-switched memory B cells (De Silva and Klein, 2015). Additionally, in BMT and mPB transplant studies, the development of naïve B cells is considered a key B cell subtype that can produce a range of antibodies, which recognise self and non-self antigens. In CBT studies, absolute counts of naïve B cells were highest within the PB of patients within the first year of transplant (Charrier et al., 2013, Servais et al., 2014). With these key roles in mind the kinetics of naïve B cell reconstitution was measured in CBT patients. Furthermore, in this current study naïve B cells have been defined as CD19+CD10+CD21+CD27-.

In the periphery and spleen, naive B cells differentiate into mature B cells upon antigenic and cytokine stimulation. The maturation of naive B cells causes loss of CD38 expression and there is continued CD24 expression on the cell surface. A CBT murine study has revealed that there is an increase in absolute numbers of mature B cells post-transplant (Wang et al., 2012b). Comparatively, this is also seen in adult human BMT studies and reconstitution of mature B cells occurred by six months post-transplant (Storek et al., 1993). Similarly, in CBT studies it has been demonstrated mature B cell reconstitution also occurs within this time frame (Renard et al., 2011, Kanda et al., 2012). With this in mind, it is important to understand the time to reconstitution of mature B cells in CBT patients. Mature B cells play a major role in infection clearance and the increased absolute counts of mature B cell post-transplant are associated with reduced incidence of aGvHD in HSCT recipients (Corre et al., 2010, Storek et al., 2001b). Furthermore, a study conducted by Weber *et al.* has shown that IL-10 produced by donor and host B cells in HSCT recipients mediate a regulatory function to suppress aGvHD after HSCT (Weber et al., 2014). Taking this into consideration, the kinetics of mature B cell reconstitution was measured in CBT patients. Furthermore, the expression of mature B cells as was defined as CD4-CD19+CD24+CD38-.

Naïve B cells can further differentiate into other B cell subsets such as activated B cells and memory B cells. These subsets can be characterised through the identification of cell surface markers such as CD21 and CD27. CD21 is a complement receptor that is expressed on B cells and is an essential marker used to identify memory B cells (Avery et al., 2005). CD27 expression aids in identifying B cell subpopulations such as activated and memory B cells as it is expressed on 40% of B cells. CD27 is highly expressed on memory B cells and has been used to specifically distinguish memory B cells from naïve B cell subtypes. Furthermore, the expression patterns of CD27 correlate with high expression levels of IgM and IgD antibodies, which shows that the cell has reached a mature state (Klein et al., 1998). Additionally, CD10 is another cell surface marker that can be used to identify B cell subsets. CD10 is commonly expressed by common lymphoid progenitors (CLP) that reside within the BM

and circulating B cells in the periphery have reduced expression of CD10. CLPs within the BM migrate either to the thymus to mature into T cells or remain within the BM to mature into B cells (Galy et al., 1995). CD10 is important in haematological diagnosis as early B cells, pro-B cells and pre-B cells express CD10. However, within this study CD10 has been used to negatively select for activated B cells, memory B cells and naïve B cells.

Through the use of surface markers such as CD21 and CD27, B cell subsets such as activated B cells, which are defined as $CD19^{+}CD10^{-}CD21^{+}CD27^{+}$, have been identified in this study. Activated B cells can be induced by APCs, which leads to somatic hypermutation and the generation of antigen specific antibodies. These antibodies can aid in the clearance of various pathogens (Agematsu et al., 1997). Interestingly, Agemastu *et al.* demonstrates that CD27+ B cells are absent in CB and the absolute counts of these cells increase with age in healthy individuals. Furthermore, CD27+ B cells can produce large numbers of Igs via direct contact with CD70 on CD4+ T cells (Agematsu et al., 1997). This is highly important in healthy individuals as well as HSCT recipients as it highlights that B cells can produce various antibodies that can distinguish self and non-self antigens. Therefore, it is important to map the kinetics of activated B cells reconstitution in CBT patients. This could demonstrate whether CBT recipients have the potential to develop antibody-producing cells that could aid in clearing infection.

Activated B cells undergo somatic hypermutation upon antigenic stimulation. This leads to the differentiation and maturation of activated B cells into memory B cells that can identify antigens upon secondary infection. Therefore, memory B cells play a fundamental role in clearance of microbes upon secondary infection. A study conducted by Thorarinsdottir *et al.* shows that CD21-CD27+ B cells are Ig class switched in healthy individuals and are antigen experienced. Furthermore, they can mount an immediate response to secondary infection (Thorarinsdottir et al., 2016). In HIV, Hepatitis C and malaria-infected individuals, the absolute numbers of memory B cells are increased. The expansion of these cells is due to chronic activation by the infective agents and auto-antigens present within the respective diseases (Charles et al., 2011, Moir

et al., 2008, Weiss et al., 2009). In HSCT, it has been demonstrated that memory B cells increase in absolute numbers post-BMT. However, the number of memory B cells is lower compared to naïve B cells (Avanzini et al., 2005). The kinetics of memory B cell reconstitution is poorly defined in CBT patients. A better understanding of the restoration of humoral immunity can be achieved by monitoring the kinetics of memory B cell reconstitution in CBT recipients. Furthermore, this will also provide an insight into the reconstitution kinetics of antibody producing B cells in CBT patients. Therefore, in this current study, the kinetics of memory B cells has been measured in CBT patients and these cells have been defined as CD19+CD10-CD21-CD27+ B cells.

Therefore in this study the kinetics and diversity of B cells in CBT patients was investigated. This was performed to further understand the time taken for B cell reconstitution post-transplant. Through this we can understand the key developmental stages involved in B cell reconstitution in CBT patients. This can provide an insight into the time in which BM output recovers and whether key developmental stages take place to reconstitute humoral immunity.

The specific aims of this chapter are to investigate the following:

1. Determine whether early reconstitution of CD19+ B cells impacts overall survival of CBT patients.
2. Map the kinetics of KREC excision circles within CBT patients to determine BM output of B cells in CBT patients.
3. Determine whether the copy number of KREC excision circles impacts the overall survival of CBT patients.
4. Map the kinetics of transitional B cell reconstitution in CBT patients.
5. Determine whether the absolute counts of transitional B cells impact the overall survival of CBT patients.
6. Map the kinetics of naïve B cell reconstitution in CBT patients.
7. Map the kinetics of mature B cell reconstitution within CBT patients.
8. Determine whether the absolute counts of mature B cells impact the overall survival of CBT patients.
9. Map the kinetics of activated B cell reconstitution in CBT patients.
10. Map the kinetics of memory B cell reconstitution in CBT patients.

5.1.1 Overall survival of cord blood transplant patients according to the CD19+ B cell count at 28 days post-transplant

In Chapter 3, it was shown that the reconstitution of CD19+ B cells takes place within 100 days post-transplant. With this in mind, it was hypothesised that the early reconstitution of CD19+ B cells may reduce infection and improve overall survival of CBT patients. Therefore, the patients were segregated into two groups, above and below the 28-day median CD19+ B cell count (n=21 patients were in each group). Landmark analysis was performed to assess whether there was a difference in the overall survival between the two groups as shown in Figure 5.1. This data demonstrates that patients with an absolute CD19+ B cell count above the median at 28 days had a two year overall survival of 87% (95% CI, 25-34%) compared to 50% (95% CI, 15-29%) for those patients with a day 28 CD19+ B cell count below the median (p= 0.04)

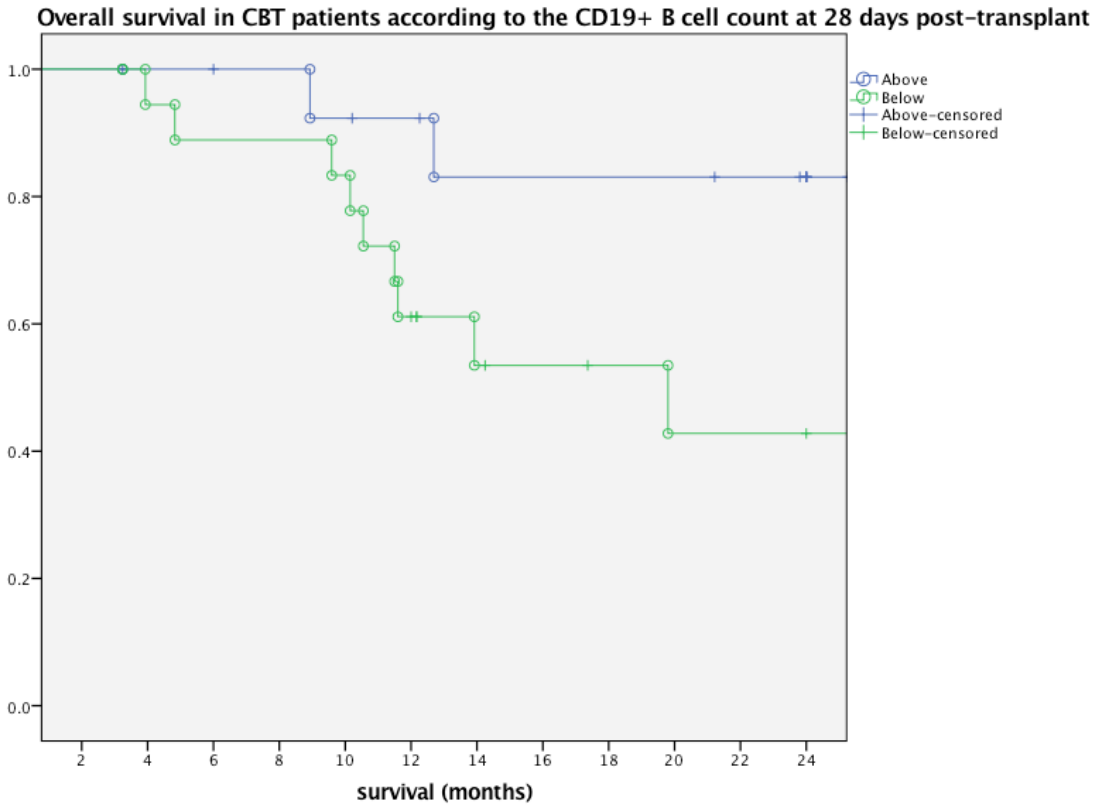


Figure 5.1. Overall survival of CBT patients according to the CD19+ B cell count at 28 days post-transplant. Kaplan-Meier curve to represent the landmark analysis for CBT patients above and below the 28-day median absolute counts of CD19+ B cells.

5.1.2 Absolute copy numbers of KRECs in cord blood transplant patients

The quantification of KREC copy numbers is a highly sensitive measure of B cell output from the BM. In this current study, the reconstitution of B cells takes place by 100 days post-transplant. With this in mind, the output of B cells from the BM was assessed via KREC quantification (Figure 5.2).

At 28 days post-CBT, the median KREC levels were significantly lower compared to healthy controls (p=0.0004). By 60 days post-CB, patients had a higher median of KREC (422517 copy number/10^7 cells) copy numbers compared to healthy controls (276393 copy number/10^7 cells). Furthermore, at 60 days post-transplant, the interquartile range for KREC copy number/10^7 cells was broader in CBT patients (0-997636 copy number/10^7 cells) compared to healthy controls (148222-421035 copy number/10^7 cells). At 100 days post-CBT, median copy number of KRECs increased and the interquartile range was broadest (602203-1189392 copy number/10^7 cells) compared to all other time points and healthy controls. By 180 days post-CBT, the median copy numbers increased in to 1091208 copy number/10^7 cells, which was significantly higher compared to all other time points and healthy controls (p=0.03). By 365 days post-CBT, the median copy number of KRECs was significantly higher compared to healthy controls (p=0.006). Additionally, the median copy number of KREC were also significantly higher compared to healthy controls at 720 days post-CBT (p=0.02). Overall, KREC copy numbers recover to the normal range by 60 days in CBT patients. This could suggest that the output of B cells from the bone marrow could recover by 60 days post-CBT.

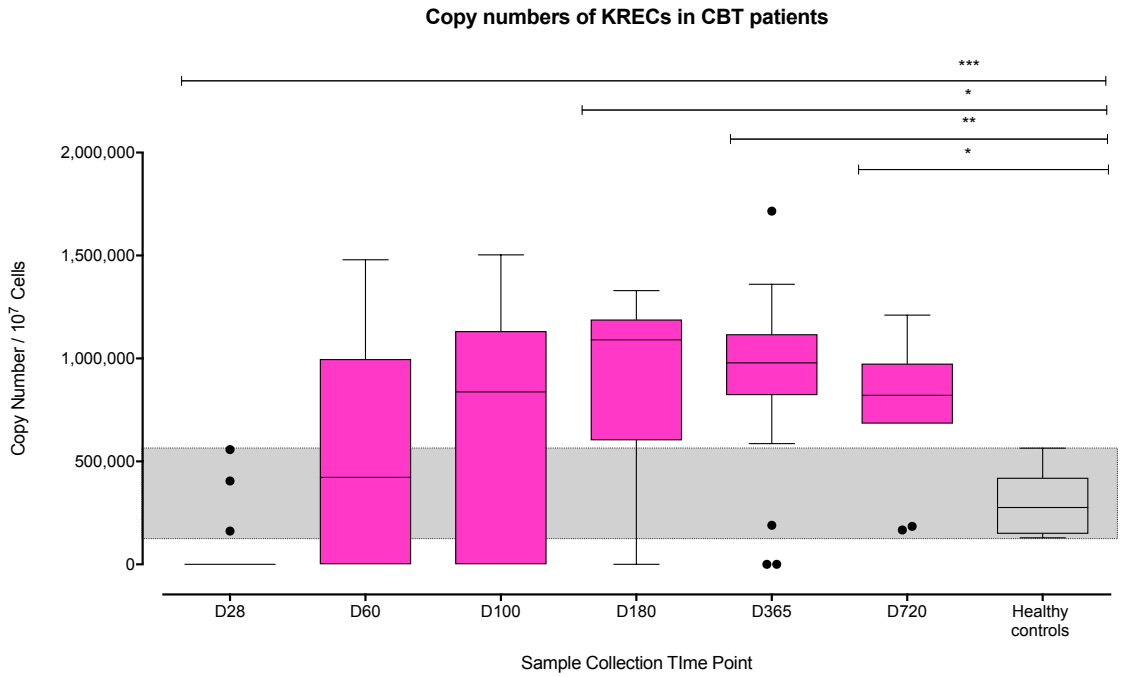


Figure 5.2 Absolute copy numbers of KRECs in CBT patients. Box and whisker plots showing the copy number of KREC genes, copy number per 10^7 Cells. A real-time PCR assay was performed to quantify KREC genes. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14. Furthermore, n = 10 healthy control samples were tested. Results are presented as a tukey analysis and a non-parametric; Mann Whitney analysis was performed between the time points and the healthy controls.

5.1.3 Overall survival of cord blood transplant patients according to the KREC copy number at 60 days post-transplant

KREC analysis specifically demonstrates that BM output of B cells recovers in CBT patients by 60 days post-transplant. It was therefore hypothesised that early reconstitution of B cells may clear infection and improve overall survival. Therefore, survival was plotted according to above and below the median 60 day KREC copy number value (n=21 patients were in each group). The 60-day value was used, as there were insufficient KREC copy numbers at 28 days to allow the analysis. To test this hypothesis landmark analysis was performed on patients who were above and below the median copy number of KRECs at 60 days post-CBT (422517 copy number/10^7 Cells), as shown in Figure 5.3.

After a follow-up time of 24 months, the probability of overall survival was 68% (95% CI, 18-31%) for patients above the median copy number of KRECs at 60 days post-CBT. Furthermore, the probability of overall survival was 75% (95% CI, 16-24%) for patients below the absolute copy number of KRECs at 60 days post-CBT. There were no major differences between the two groups in terms of overall survival.

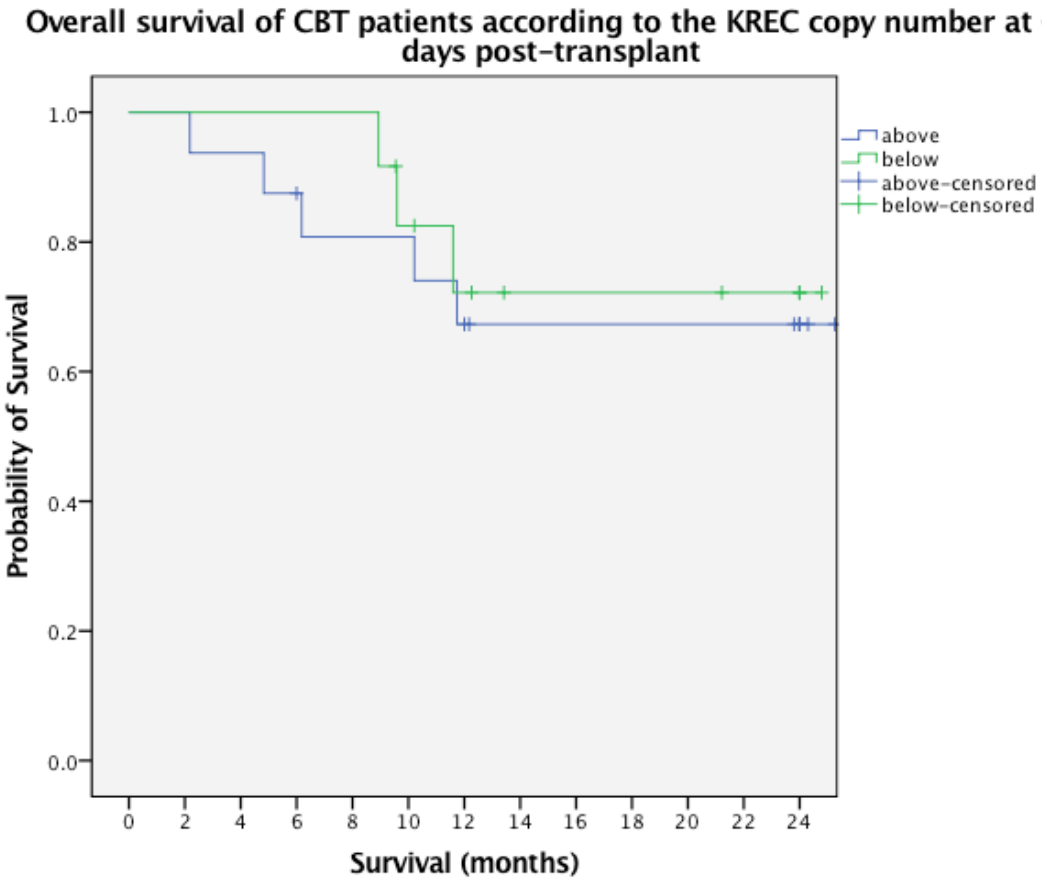


Figure 5.3 Overall survival of CBT patients according to the KREC copy number at 60 days post-transplant. The graph above is a Kaplan-Meier curve for the overall survival of CBT patients above and below the 60-day median copy number of KRECs**.**

5.1.4 Transitional B cell count recovery following allogeneic cord blood transplantation

As our results indicate that there is rapid reconstitution of CD19+ B cells in CBT patients, the diversity of B cell subsets and their patterns of reconstitution in CBT patients were further investigated. The reconstitution patterns of CD19+ transitional B cells were identified in CBT patients. These cells are defined as CD4-CD19+CD24+CD38^{high}, as shown in Figure 5.4.

At 28 days, the absolute median count of transitional B cells (0 cells/μl, range: 0-45 cells/μl) in CBT patients was significantly lower compared to healthy control PB (12 cells/μl) (p=0.0001). The median absolute counts at 60 days were similar to the 28 day median count post-transplant. However, by 100 days post-transplant, the absolute median counts of transitional B cells increased to 35 cells/μl, which was higher compared to healthy control PB. Furthermore, the interquartile range was broader in CBT patients (0-130 cells/μl) at 100 days compared to healthy control PB (6-24 cells/μl). At 180 days post-CBT, the absolute median counts of transitional B cells remained at similar levels to healthy controls. However, the interquartile range was broader in CBT patients (0-259 cells/μl) compared to healthy control PB. Furthermore, the interquartile range was broadest at 180 days in CBT patients compared to all other time points. By 365 days post-transplant, the absolute median levels of transitional B cells increased to 89 cells/μl, which was significantly higher compared to healthy control PB (p=0.0005). Subsequently, the absolute median levels decreased to 40 cells/μl at 720 days. However, this was significantly higher compared to healthy control PB (p=0.03). Overall, this data suggests that reconstitution of transitional B cells takes place by 100 days post-transplant and the absolute median counts of transitional B cells remain higher compared to healthy controls between 100 and 720 days post-CBT.

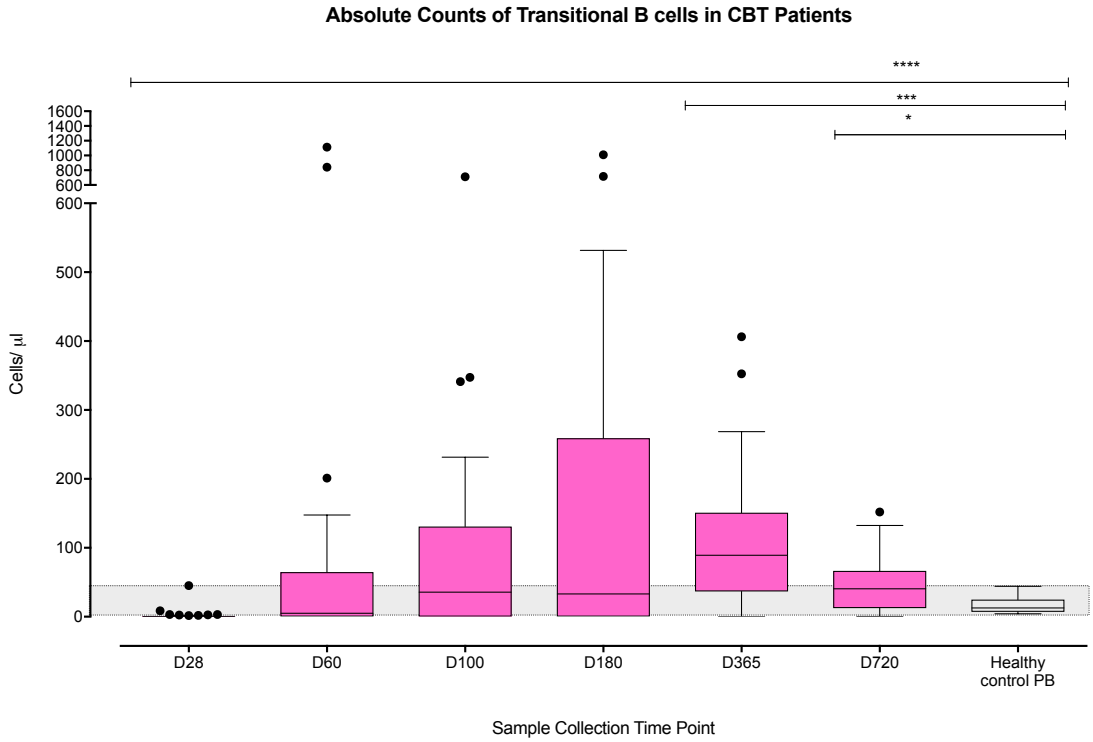


Figure 5.4 Absolute counts of transitional B cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of transitional B cells (CD4-CD19+CD24+CD38^{high}) in CBT patients. Flow cytometry was performed to quantify the absolute counts of transitional B cells (CD4-CD19+CD24+CD38^{high}). At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14. Furthermore, n=10 healthy control PB samples were also tested. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls, p<0.05 (*), p<0.001 (***) and p<0.0001 (****).

5.1.5 Overall survival of cord blood transplant patients according to the absolute transitional B cell number at 60 days post-transplant

As previously shown, reconstitution of transitional B cells occurs by 100 days post-CBT. Given the survival difference according to the absolute CD19+ B cell count at 28 days, it was hypothesised that the recovery of transitional B cells differentiate into mature B cells and may reduce infection and improve overall survival. To test this hypothesis, patients were segregated into two groups, above and below the 60-day transitional B cell median (n=21 patients were in each group). Survival was plotted according to the absolute transitional B cell median at 60 days post-CBT (4 cells/μl) (Figure 5.5). The day 60 transitional B cell value was used, as there were insufficient transitional B cells at 28 days post-transplant to allow this analysis.

The probability of overall survival was 71% (95% CI, 20-31%) at 2 years post-transplant for patients above the 60 day transitional B cell absolute median count. The probability of overall survival was 73% (95% CI, 26-42%) for patients below the 60-day transitional B cell absolute median count. Overall, this suggests that the early reconstitution of transitional B cells does not impact on the overall survival of CBT patients post-transplant.

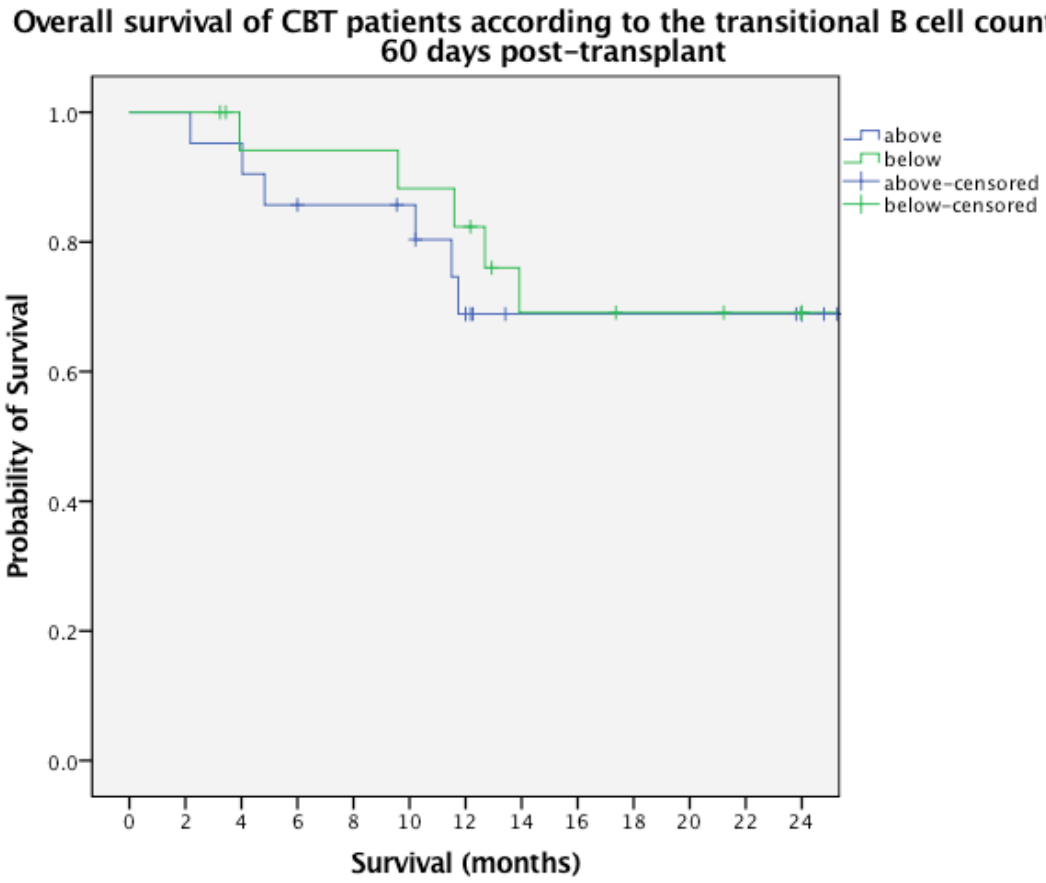


Figure 5.5 Overall survival of CBT patients according to the transitional B cell number at 60 days post-transplant. A Kaplan-Meier curve for the overall survival of CBT patients above and below the median for the 60-day transitional B absolute count.

5.1.6 Naïve B cell count recovery following allogeneic cord blood transplantation

Naïve B cells are one of the early B cells released into the PB system and are antigen inexperienced. As previously described, upon antigenic stimulation these cells differentiate into mature B cells to produce antigen specific antibodies. With this in mind, the reconstitution of naïve B cells has been identified in CBT patients (Figure 5.6). These cells have been defined as CD19+CD10+CD21+CD27-.

In CBT patients at 28 days, the absolute median level (0 cells/μl, range 0-38 cells/μl) of naïve B cells was significantly lower compared to healthy control PB (113 cells/μl) (p=0.0001). The absolute median levels in CBT patients remained lower compared to healthy control PB up to 100 days post-transplant. At 100 days post-CBT, the absolute median level of naïve B cells was 118 cells/μl. Furthermore, the interquartile range in CBT patients (10-531 cells/μl) at 100 days was broader compared to healthy control PB (64-231cells/μl). The absolute median levels of naïve B cells increased to 193 cells/μl at 180 days post-CBT, which was higher compared to healthy control PB (113 cells/μl). The highest absolute median level of naïve B cells was recorded at 365 days post-transplant (660 cells/μl) and was significantly higher compared to healthy control PB (p=0.04). By 720 days, the absolute median levels of naïve B cells decreased to 263 cells/μl. However, absolute median counts of transitional B cells were higher compared to healthy control PB (113 cells/μl). The broadest interquartile range (132-1537 cells/μl) was recorded at 720 days post-CBT and was broader compared to healthy control PB and all other time points. Overall, the absolute numbers of naïve B cells reach the healthy control range by 100 days post-CBT and then continue to rise beyond that for up to 720 days post-transplant. This suggests that the reconstitution of naïve B cells occurs by 100 days.

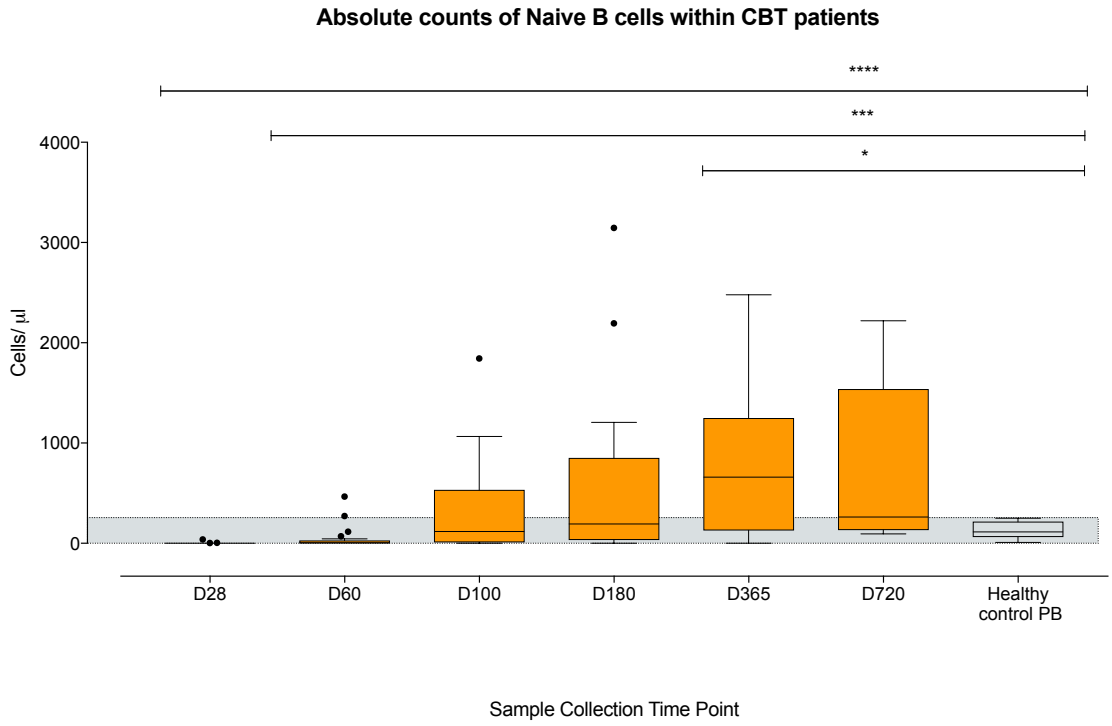


Figure 5.6 Absolute counts of naive B cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter of naïve B cells within CBT patients in the UK. Flow cytometry was performed to quantify naive B cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26 and D720 = 14. Furthermore, n=10 healthy control PB patient samples were acquired. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls, $p<0.05$ (*) and $p<0.001$ (***).

5.1.7 Mature B cell count recovery following allogeneic cord blood transplantation

As our results indicate that there is reconstitution of CD19+ B cells and transitional B cells in CBT patients by 100 days post-CBT, the reconstitution pattern of mature B cells in CBT patients were further investigated. These cells are defined as CD4-CD19+CD24+CD38^{low} as shown in Figure 5.7.

At 28 days post-CBT, the median absolute counts of mature B cells within CBT patients (12 cells/μl) were significantly lower compared to healthy control PB (148 cells/μl) (p=0.0001). By 60 days post-CBT, the median absolute counts increased to 10 cells/μl, which was lower compared to healthy control PB. By 100 days post-CBT there was an increase in the median absolute counts of mature B cells in CBT patients (145 cells/μl) and the interquartile range (0-667 cells/μl) was broader compared to healthy control PB (86-269 cells/μl). At 180 days the median absolute count increased to 366 cells/μl. This was higher compared to healthy control PB and the broadest interquartile range was also observed at this time point in CBT patients (0-1896 cells/μl) Successively, at 365 days the median absolute count remained higher (870 cells/μl) compared to healthy control PB and the interquartile range (333-1802 cells/μl) was also broader compared to healthy control PB (p=0.0001). At 720 days post-CBT, the median absolute levels (443 cells/μl) remained higher compared to healthy control PB and the interquartile range was also broader (336-1483 cells/μl) compared to healthy control PB (p=0.0007). Overall, this data suggests that the reconstitution of mature B cells takes place by 100 days post-transplant. Absolute median levels of mature B cells are higher compared to healthy control PB from 180 days to 720 days post-CBT. The increase in mature B cell numbers could be due to the fact that there is maturation of naïve B cells by 720 days post-transplant. Therefore, this could suggest that there is restoration of humoral immunity in CBT patients, which takes place by 720 days post-transplant.

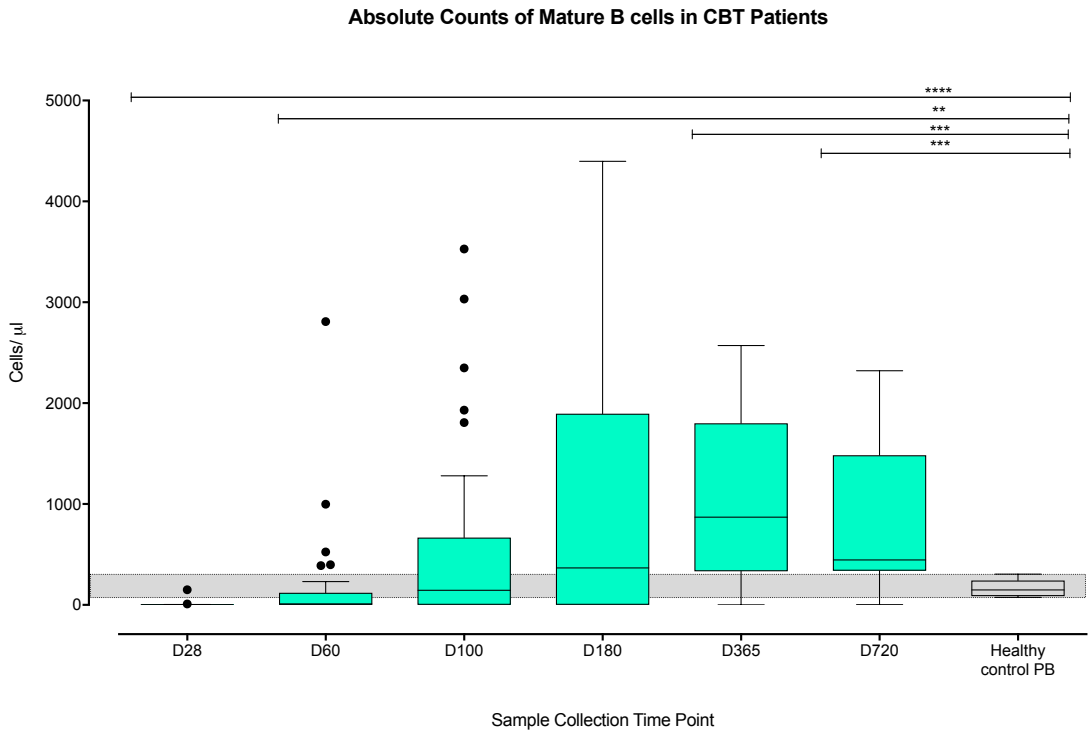


Figure 5.7 Absolute counts of mature B cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter of mature B cells within CBT patients. Flow cytometry was performed to quantify mature B cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26 and D720 = 14. Furthermore, n=10 healthy control samples were acquired. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls, $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).

5.1.8 Overall survival of cord blood transplant patients according to the mature B cell count at 60 days post-transplant.

In this current study, it has been demonstrated that the reconstitution of mature B cells within CBT patients takes place by 100 days post-transplant. Given the survival difference according to the absolute CD19+ B cell count at 28 days, it was hypothesised that the recovery of mature B cells may reduce infection and improve overall survival in CBT patients. Survival was plotted according to above and below the 60-day median absolute count of mature B cells. The 60-day value was used, as there were insufficient mature B cells at 28 days to allow this analysis. To test this hypothesis, landmark analysis was performed on patients who were above (n=21) and below (n=21) the 60-day absolute median count of mature B cells (10 cells/μl), as shown in Figure 5.8.

After a follow-up time of 24 months, the probability of overall survival was 79% (95% CI, 22-32%) for patients above the 60-day mature B cell absolute median count. Furthermore, the probability of overall survival was 67% (95% CI, 22-39%) for patients below the 60-day mature B cell absolute median count. Overall, this suggests that the early reconstitution of mature B cells does not impact the overall survival of CBT patients post-transplant.

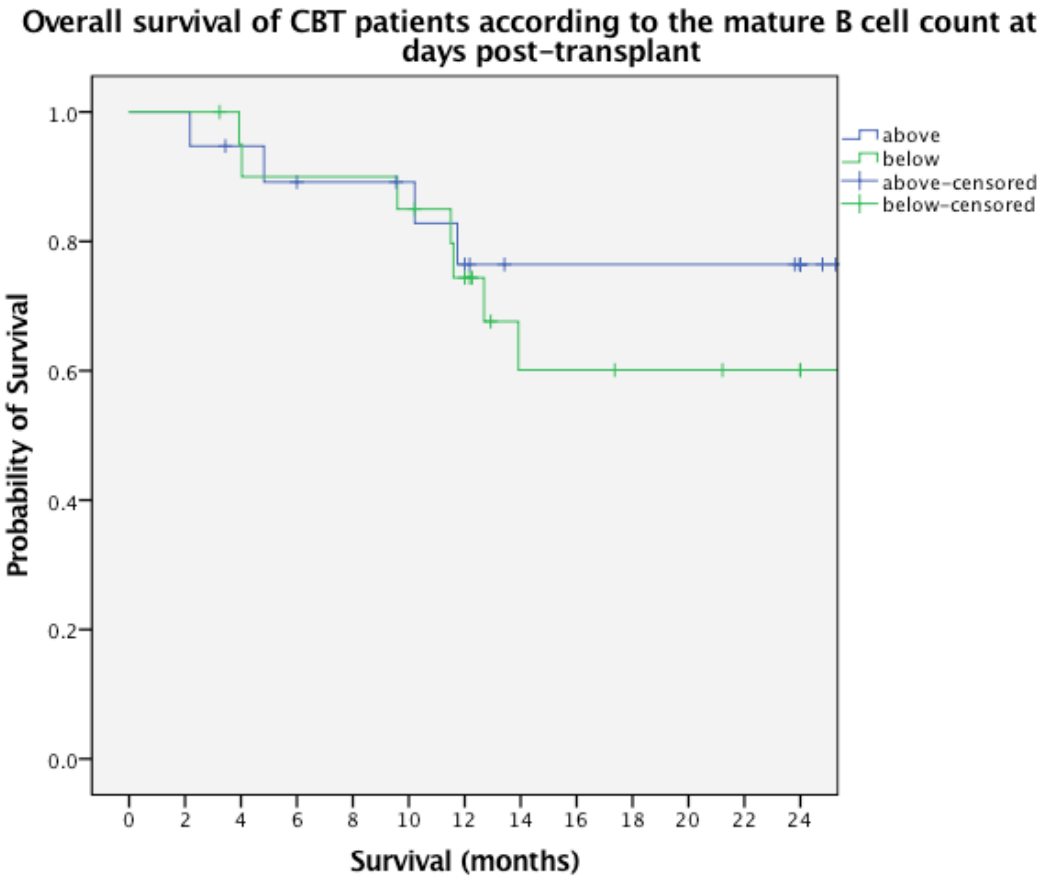


Figure 5.8 Overall survival of CBT patients according to the mature B cell count at 60 days post-transplant. Kaplan-Meier curve for overall survival in CBT-patients above and below the 60-day absolute count median of mature B cells.

5.1.9 Activated B cell count recovery following allogeneic cord blood transplantation

Activated B cells are an essential component of humoral immune responses. They are stimulated by the presentation of pathogenic antigens, which leads to their maturation and the generation of antigen specific antibodies. With this in mind, we have mapped the kinetics of reconstitution of activated B cells in CBT patients, which have been defined as CD19+CD10-CD21+CD27+ (Figure 5.9).

At 28 days post-CBT, the absolute median counts (0 cells/μl range: 0-44 cells/μl) of activated B cells were significantly lower in CBT recipients compared to healthy control PB (26 cells/μl) ($p=0.0002$). Sequentially, at 60 days post-transplant the absolute median counts were also significantly lower compared to healthy control PB absolute median levels ($p=0.001$). At 100 days post-CBT, absolute median levels increased to 12 cells/μl. However, median levels were still below the healthy control PB levels. At 180 days post-CBT, the absolute median levels of activated B cells (33 cells/μl) were higher compared to healthy control PB (26 cells/μl). The absolute median levels remained constant at 365 days post-CBT and the highest median levels were measured at 720 days post-CBT (104 cells/μl) compared to healthy control PB (26 cells/μl). The broadest interquartile range was measured at 720 days post-transplant in CBT patients (17-244 cells/μl) compared to healthy control PB (18-73 cells/μl). Overall, this data suggests that there is an increase in the absolute count of activated B cells in CBT patients. Furthermore, the absolute numbers of activated B cells reach the healthy control range by 180 days and then continue to rise beyond that for up to 720 days post-transplant. This could be due to maturation of transitional and naïve B cells between 100 and 180 days post-CBT. Antigens could stimulate these B cell subtypes by 100 days and this could induce their development into activated B cells.

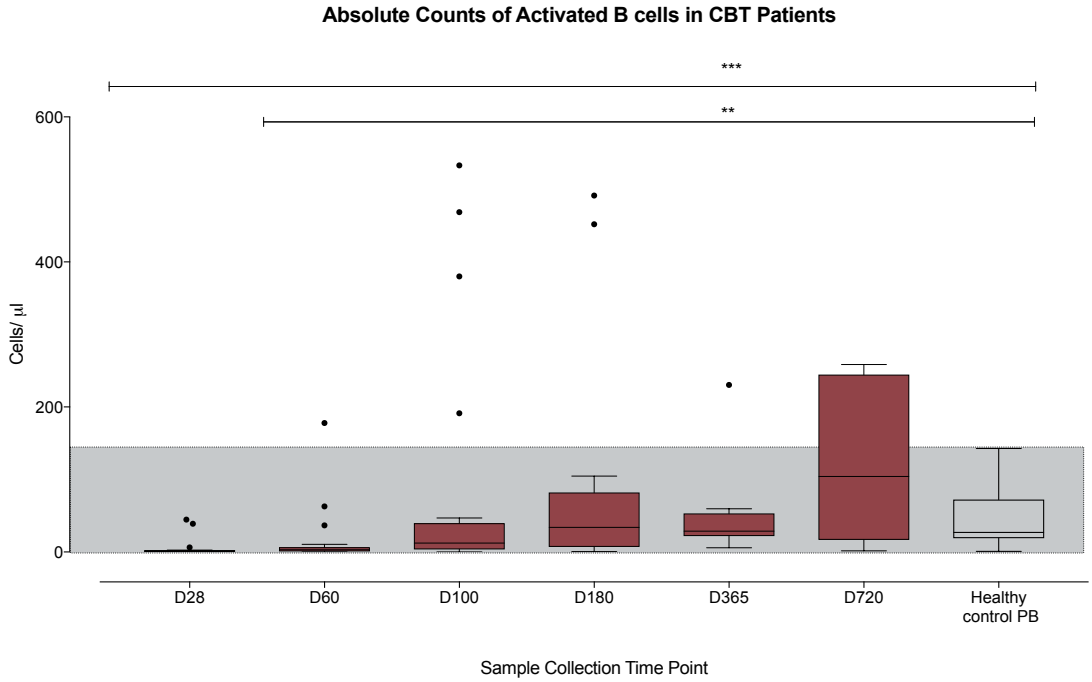


Figure 5.9 Absolute counts of activated B cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter of activated B cells within CBT patients. Flow cytometry was performed to quantify activated B cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26 and D720 = 14. Furthermore, n=10 healthy control PB samples were acquired. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls, $p<0.01$ (**) and $p<0.001$ (***).

5.1.10 Memory B cell count recovery following allogeneic cord blood transplantation

Memory B cells are an essential cell type in humoral immune reactions. Upon antigenic stimulation, activated B cells develop into memory B cells. The development of memory B cells demonstrates that long term B cell immunity takes place, which leads to specific antibody production against foreign pathogens. Furthermore, it also demonstrates that B cells could develop a broad BCR repertoire to distinguish a variety of antigens. Restoration of long-term humoral immunity is vital for the clearance of infection and long-term management of secondary infections that may arise in CBT patients. In this study, memory B cells are characterised as CD19+CD10-CD21-CD27+ and the kinetics of reconstitution was investigated in CBT patients (Figure 5.10).

At 28 days post-transplant, the absolute median count of memory B cells was significantly lower in CBT patients (0 cells/μl) compared to healthy control PB (30 cells/μl) ($p=0.0001$). Sequentially, at 60 days post-CBT the absolute median count of memory B cells was also significantly lower in CBT patients compared to healthy control PB ($p=0.0001$). The absolute median count of memory B cells remained below healthy control PB for up to 100 days. At 180 days post-CBT the absolute median levels of memory B cells in CBT patients (33 cells/μl) increased and were equivalent to healthy control PB (30 cells/μl). Respectively, the interquartile range (15 – 288 cells/μl) is broadest at 180 days post-CBT compared to healthy control PB (20 – 52 cells/μl) and all other time points. The absolute median counts increased and were highest at 365 days post-transplant (95 cells/μl), which was significantly higher compared to healthy control PB ($p=0.03$). However, by 720 days post-transplant, the absolute median levels of memory B cells were reduced in CBT patients to 31 cells/μl, which was equivalent to the absolute median levels within healthy control PB. Overall, this data suggests that memory B cell reconstitution takes place by 100 days post-transplant. Furthermore, it can be seen that there is an increase in the absolute number of memory B cells by 365 days post-CBT and a decrease

in activated B cells by the same time point. This could suggest that activated B cells are maturing into memory B cells by 365 days post-CBT.

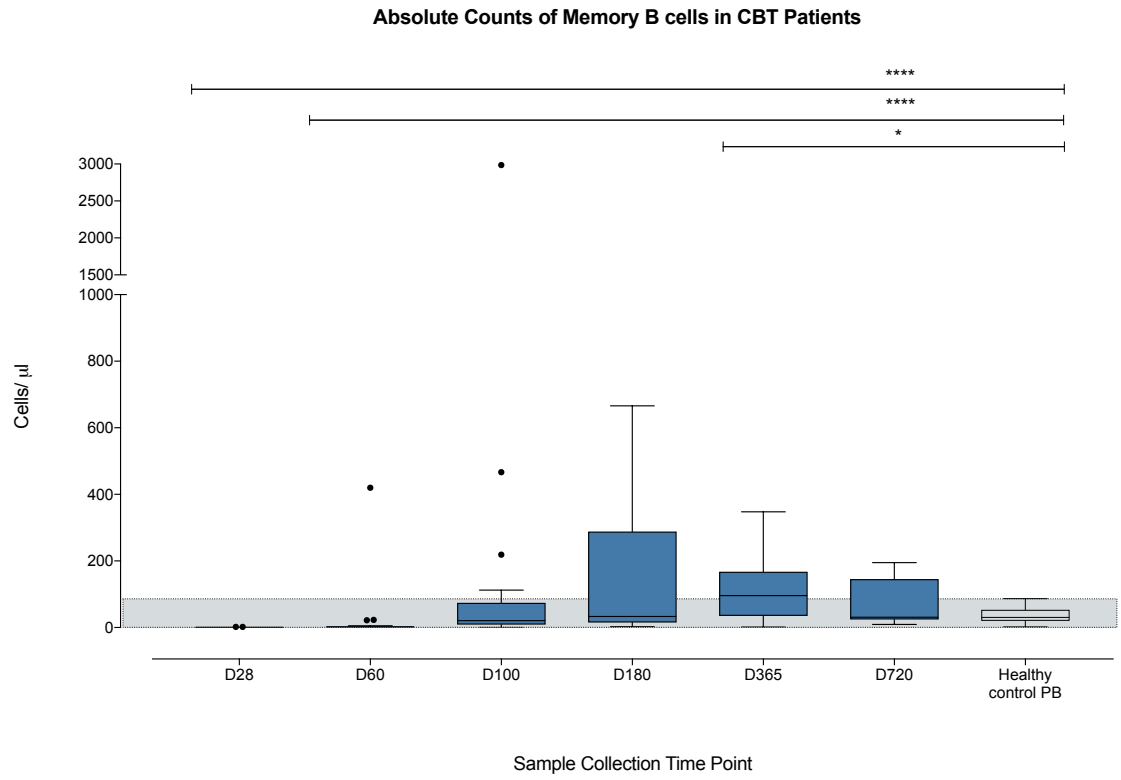


Figure 5.10 Absolute counts of memory B cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter of memory B cells within CBT patients. Flow cytometry was performed to quantify memory B cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26 and D720 = 14. Furthermore, n=10 healthy control PB samples were acquired. Result are presented as a tukey analysis and a non-parametric, Mann Whitney analysis was performed between the time points and the healthy controls, $p<0.01$ (**) and $p<0.0001$(****).

5.2 Discussion

As far as we are aware, the current study is one of the first studies to investigate the diversity and kinetics of B cell reconstitution in CBT patients within the UK. As previously described in Chapter 3, section 3.3, it has been shown that CD19+ B cell reconstitution takes place by 100 days post-transplant. Data from this current study corroborates with other CBT immune reconstitution studies that also show this pattern of B cell reconstitution (Jacobson et al., 2012, Kanda et al., 2012, Beaudette-Zlatanova et al., 2013). However, to extend upon published data, this current study identifies the reconstitution of a broad subset of B cell populations (activated B cells, naïve B cells and memory B cells) in CBT patients. By measuring the reconstitution patterns of B cells subsets, the kinetics and diversity of B cells were defined in CBT patients.

In this study, CBT patients with an absolute count above the 28-day median of CD19+ B cells have a better overall survival compared to patients below median CD19+ absolute count. As far as we are aware, there are no other CBT studies that have demonstrated this even though other studies have shown early reconstitution of B cells in CBT patients. However, it has been reported in severe combined immunodeficiency patients that long-term survival is improved in patients with higher CD19+ B cell absolute counts after BMT (Lev et al., 2012). Additionally, in paediatric studies, immune reconstitution kinetics has been used as an early predictor for mortality. However, B cells have not been used in survival analysis even though there is rapid reconstitution of B cells within this patient cohort (Bartelink et al., 2013). With this in mind, it is important to investigate the overall survival of HSCT patients and future immune reconstitution studies should aim to correlate overall survival with B cell reconstitution as they could reduce infection and improve overall survival.

Through the use of immunophenotyping methods it has been demonstrated that there was early reconstitution of CD19+ B cells in CBT patients. This led to the development and testing of specific gene segments found in naïve/transitional B cells, known as KRECs. Identification of these gene excision circles was performed, as it is a sensitive measure to quantify the output of B cells from the

BM in CBT recipients. This method was used to measure the kinetics of naïve and transitional B cell reconstitution in CBT patients. KRECs are bi-product excision circles that are excised through the gene re-arrangements in the Ig locus of B cells during B cell maturation within the BM (Siminovitch et al., 1985, van Zelm et al., 2007). In this current study, absolute copy numbers of KRECs increase in CBT patients between 28 and 720-days post-transplant. In the median number of patients (n=21) at 60 days, KREC copy numbers reached the healthy normal range. Therefore data from this current study corroborates data from a study conducted by Nakatani *et al.* who demonstrated that B cell reconstitution occurs by 100 days post-transplant. Furthermore, Nakatani *et al.* also showed that high KREC levels are associated with a decrease frequency of infectious episodes in CBT patients (Nakatani et al., 2014). Unfortunately, infection data was not collected for this current study, as it was incomplete and correlations between infection and KREC copy numbers could not be made. KREC copy numbers have also been measured in a BMT study of RAG-deficient SCID patients. These patients had an increase in absolute copy numbers of KRECs after BMT. In these patients, B cell reconstitution occurs within the first six months post-transplant (Lev et al., 2012). There is an increasing amount of data that confirms that there is a rise in absolute KRECs post-HSCT and this is associated with enhanced B cell neogenesis. Data from this current study also corroborates that there is a rise in KREC levels in CBT patients post-transplant. (Sottini et al., 2014, Serana et al., 2010, Sottini et al., 2012, Mensen et al., 2013, Mensen et al., 2014).

Herein, we have shown that there is an increased number of KRECs in CBT patients for up to 720 days post-transplant. Furthermore, the number of KRECs is higher compared to healthy controls. With this in mind, it was hypothesised that the absolute number of KRECs could impact overall survival of CBT patients. To test this hypothesis, patients were separated into two groups, above and below the 60-day KREC median copy number and a landmark analysis was performed. The 60-day median copy number was used, as there were insufficient KREC copy numbers at 28-days post-CBT to perform this analysis. The analysis showed that there is no difference in survival between the two groups. As far as we are aware, similar survival analysis has not been

performed in HSCT studies, comparing KREC levels with overall survival. However, as B cells are one of the first lymphocytes to reconstitute in CBT patients after NK cells, it could be believed that they could play a major role in reducing post-transplant complications such as infection and GvHD. Through this analysis, we believe that future studies in immune reconstitution should assess the impact of increased KREC levels and should be correlated with clinical outcomes post-transplant. This could provide a better understanding as to whether early B cell reconstitution impacts clinical outcomes such as GvHD and incidence of infection. Again, this could not be correlated in this current study, as the GvHD and infection data was not complete.

Transitional B cells and immature B cells are the first B cell subtype to be released from the BM upon B cell maturation. This current study shows that transitional B cells (CD4-CD19+CD24+CD38+) reconstitute by 60 days post-transplant. In human development, transitional B cells are the first to arise before the mature B cell subtype and are considered an important intermediate for the generation of mature B cells. In a study conducted by Marie-Cardine *et al.* it has been demonstrated that transitional B cells are the first subtype of B cells that reconstitute within BMT recipients. This is followed by an increase in absolute numbers of mature B cells. However, as absolute numbers of mature B cells increase, absolute numbers of transitional B cells decrease (Marie-Cardine et al., 2008). This suggests that B cells are maturing within CBT patients and the data that we have presented within this project corroborates with this.

The expansion of transitional B cells has also been reported in a number of other diseases such as: HIV patients with advanced disease; patients with idiopathic CD4 T cell lymphocytopenia; X linked lymphoproliferative syndrome and patients with SLE (Malaspina et al., 2006, Cuss et al., 2006, Sims et al., 2005, Malaspina et al., 2007). This shows transitional cells increase in immunodeficient and autoimmune patients and they could be an essential cell type to develop into mature B cells. The increase in absolute B cell numbers could be a compensatory mechanism to overcome lymphopenia in HIV and CD4 T cell lymphopenia patients. Therefore, absolute B cell numbers could

increase, as they are required to fill a cell niche. Similarly, this theory could be applied to CBT patients, as there is delayed reconstitution of T cells post-transplant and B cells could expand and proliferate to compensate for the reduced numbers of T cells. Furthermore, the expansion of transitional B cells within CBT patients could occur due to factors addressed in Chapter 3, Section 3.3.

It is likely that the development of transitional B cells is a critical checkpoint in B cell maturation. However, if the development of B cells breaks down and the expansion of transitional B cells does not take place, autoimmune diseases such as rheumatoid arthritis and SLE could develop (Samuels et al., 2005, Yurasov et al., 2005). It could also be likely that the development of GvHD in HSCT patients is due to the breakdown in tolerance of B cell development. Early post-transplant, transitional B cells have a low BCR diversity, which could lead to the production of antibodies that induce the development of GvHD (Kier et al., 1990, Patriarca et al., 2006). With this in mind, the development of transitional B cells and the regulation of B cell tolerance are essential post-transplant to prevent the development of GvHD in CBT patients.

In this project, the kinetics of naïve B cell reconstitution was investigated to understand whether there is restoration of a key developmental stage in B cell maturation. Naïve B cells are a key developmental point in healthy adults as they arise from transitional B cells, which develop within the spleen upon antigen stimulation (De Silva and Klein, 2015). In this project, data shows that naïve B cell numbers increase in CBT patients and absolute counts are higher compared to healthy controls. Respectively, the highest absolute levels of naïve B cells are seen in CBT patients at 365 days post-transplant. This corroborates data seen within HSCT studies, where there were higher absolute numbers of naïve B cells within the first year of transplant (Charrier et al., 2013, Servais et al., 2014). Therefore, this highlights that naïve B cell reconstitution takes place by 365 days post-CBT and this fundamental maturation stage leads to mature B cell formation.

Mature B cells arise from the maturation of naïve and transitional B cells, which occurs through antigenic challenge. This takes place in secondary lymphoid organs. In the secondary lymphoid organs, mature B cells express IgM and IgD antibodies. Mature B cells can recirculate between the blood and lymphoid organs and can enter B cell follicles within lymph nodes and the spleen. In these microenvironments, B cells encounter T cells, which induces B cell maturation and antibody production (Klein et al., 1998). With these important roles in mind, the kinetics of mature B cell reconstitution was measured in CBT patients. The highest absolute count of mature B cells was measured at 365 days post-CBT. This supports the data demonstrated by Marie-Cardine *et al.* who displays that there an increase in the number of mature B cells in HSCT patients, which occurs within the first year post-transplant (Marie-Cardine et al., 2008). In this current study, there is reconstitution of mature B cells in CBT patients by 100 days post-transplant. Furthermore, over the 720-day follow up period, it has been shown that there is an increase in the absolute number of mature B cells whilst there is a decrease in the absolute numbers of transitional B cells. As previously described, the development of mature B cells arises through maturation and proliferation of transitional B cells. Therefore, our data could be demonstrating that transitional B cells are maturing and undertaking a mature B cell phenotype over the 720-day follow up. As it has been shown that that there is a rapid reconstitution of mature B cells in CBT patients, it was hypothesised that early reconstitution of mature B cells could impact overall survival of CBT patients. However, through this analysis it was seen that the overall survival is not impacted by higher or lower absolute levels of mature B cells at 28 days post-CBT.

In the spleen and germinal centre of lymph nodes, B cells can become activated through antigen presentation via APCs. This leads to increased expression of CD27 and antibody expression such as IgM and IgD. The activation of B cells is an important stage in humoral responses as B cells undergo somatic hypermutation and produce antigen specific antibodies (Agematsu et al., 1997). Herein, it has been shown that activated B cells reconstitute by 100 days post-CBT.

Furthermore, activated B cells can mature into memory B cells and it has been demonstrated in this study that memory B cells reconstitute by 100 days post-CBT. However, the levels of memory B cells remain low up to 180 days post-CBT and reconstitution of memory B cells takes place by 365 days post-transplant. This also corroborates HSCT studies where it has been observed that memory B cell reconstitution occurs by one year post-transplant (Avanzini et al., 2005). A reason as to why memory B cells develop by 365 days post-transplant could be related to the fact that many CBT patients are vaccinated post-transplant. Generally in the UK, patients are vaccinated between six months and two years post-transplant (Ljungman et al., 2009). Therefore, vaccination could cause an increase in memory B cells post-transplant, particularly at one year post-transplant. This increase in memory B cells could be due to vaccines causing antigenic stimulation of B cells and maturation of naïve B cells. This subsequently leads to an increased memory B cell subset post-transplant.

Additionally, the development and expansion of memory B cells has also been associated with infectious episodes such as EBV, which is a common opportunistic infection in HSCT patients and will infect resting B cells. This can lead to the activation of resting B cells, causing them to proliferate. It has also been shown that memory B cells are enriched with regulatory B cells and these are present in healthy individuals. However, HSCT patients who develop cGvHD have been shown to lack memory B cells that produce IgM and a have a reduction in regulatory B cells numbers (Khoder et al., 2014). Respectively, these memory B cells have been shown to produce IL-10 and can suppress immune reactions. Therefore, this highlights a key role for memory B cells as they could express and secrete IgM antibodies. Additionally, they could play an immunomodulatory role with regulatory B cells that leads to less severe cGvHD (Sarvaria et al., 2016). This suggests that maturation of B cells is important in HSCT patients, however further functional maturation of B cells is also crucial in HSCT to prevent development of severe GvHD.

Overall, this current study shows that there is early reconstitution of B cells in CBT patients and that there is reconstitution of the different B cell

subpopulations post-transplant. B cells undergo a various maturation stages and these subsets reconstitute in CBT patients, which could lead to restoration of humoral immunity. However, further analysis is required to understand the functionality of B cells in CBT recipients. Additionally, studies of BCR specificity and diversity are required to understand how broad the BCR repertoire is post-CBT. BCR spectratyping was not performed in this current study and could have provided an insight into the restoration of B cell specificity for antigens. Additionally, measuring antibody titre levels for IgM and IgD could have also provided an indication of B cell maturation in CBT recipients. Functional studies are required to understand whether B cells also play a functional role in CBT patients and whether they aid in the clearance of infection and reduce the incidence of cGvHD.

Chapter 6 : T cell reconstitution and diversity in cord blood transplant patients

6.1 Background and Aims

T cells are an essential component of the adaptive immune system and play a pivotal role in inducing cell-mediated responses against foreign pathogens. These cells also have a crucial role in inducing GvHD and the rapid reconstitution of T cells are known to play a role in aGvHD development (Sakoda et al., 2007). However, T cells are also required for an optimised GvL response and could reduce the incidence of relapse (Barrett, 2008).

HSCT recipients are severely immunocompromised post-transplant and experience delayed immune reconstitution, which increases the risk of infectious complications and this compromises tumour immunosurveillance (Maury et al., 2001). Delayed T cell reconstitution can occur due to T cell depletion from the graft and thymic damage caused by conditioning prior to HSCT (Douek et al., 2000). The development of GvHD can further exacerbate a delay in T cell reconstitution, which can increase the risk of life threatening viral, bacterial and fungal infections (Storek et al., 2002). Notably, in Chapter 3, it was shown that the reconstitution of CD3+, CD4+ and CD8+ T cells are delayed in CBT patients. CD3+ and CD4+ T cell reconstitution could take longer than 720 days and CD8+ T cell reconstitution occurs by 365 days post-transplant. However, the reconstitution patterns of T cell subsets in CBT patients required further assessment and have been addressed in this chapter.

T cell reconstitution can occur through two pathways known as the thymic dependent and thymic independent pathway. The thymic dependent pathway occurs when naïve T cells are being produced within the thymus from CLPs. However, thymic independent reconstitution occurs where mature T cells from the graft expand and proliferate within the recipient (Williams et al., 2007).

Thymopoiesis is the production of naïve T cells from the thymus and it begins in childhood and continues into adulthood. However, the level of thymopoiesis varies in adulthood. The recovery of thymopoiesis after HSCT is an important determinant of naïve T cell output from the thymus and the level of T cell receptor diversity (Komanduri et al., 2007). Thymic output can be determined in HSCT patients via measurement of the expression patterns of cell surface markers such as CD31 (Douek et al., 2000). CD31 is a cell surface marker that aids in identifying naïve T cells that have recently egressed from the thymus (Kohler and Thiel, 2009). These T cells are known as recent thymic emigrants (RTEs). CD31+ (CD4+ or CD8+) T cells contain a high number of T cell receptor excision circles (TRECs), which are excision circles of DNA of the delta locus and become excised during rearrangement of the T cell receptor gene (Ribeiro and Perelson, 2007). Approximately 70% of newly produced T cells within the thymus contain TRECs and in adults there is a decline in the number of TRECs due to thymic involution (Lynch et al., 2009). On the other hand, TRECs can still be detected within T cells of elderly patients and this demonstrates that the thymus still has a functional component irrespective of age (Douek et al., 1998, Douek et al., 2000). In this current study, the median age of patients was 48 years old and as patients were older they could have reduced thymic output due to thymic involution. Factors that contribute to a delay in T cell reconstitution include: patient age, conditioning and chemotherapy and post transplant immunosuppression. (Lynch et al., 2009). With this in mind, it was hypothesised that thymic output of naïve T cells will be delayed in this cohort of CBT patients. To test this hypothesis, TREC gene copy numbers were quantified to measure the output of naïve T cells from the thymus in CBT patients. Furthermore, the absolute quantification of RTEs (CD31+CD45RA+) via immunophenotyping was performed as an alternative measure of thymic output in CBT patients.

Complete T cell reconstitution is also dependent on peripheral expansion of T cells. This takes place within the peripheral blood system where naïve T cells differentiate into mature T cells upon antigenic stimulation. To better understand the reconstitution of specific T cell subsets, surface markers such as CD45RA and CCR7 have been used (Sallusto et al., 2014). The combination of CD45RA

and CCR7 can define T cell subsets such as: naïve (CD45RA+CCR7+), central memory T cells (CD45RA-CCR7-), effector T cells (CD45RA-CCR7+) and effector memory T cells (CD45RA+CCR7-).

Within the periphery, naïve T cells migrate to secondary lymph nodes where a number of antigens will be presented by APCs (Butcher and Picker, 1996). The migration of naïve T cells to secondary lymph nodes is mediated through the expression of chemokine receptors such as CCR7. T cells expressing CCR7 are able to enter secondary lymphoid tissues via the high endothelial venules. Ligands for CCR7 are expressed by high endothelial venules of secondary lymphoid organs, parenchymal cells within T zones of lymph nodes and by endothelial cells at the openings of the lymphatic vessels within peripheral tissues (Gunn et al., 1998). At these sites, APCs will promote the maturation of naïve T cells into effector, effector memory T cells and, subsequently, central memory T cells. CCR7+ T cells can be defined as effector T cells as they express lymph node homing receptors and lack an immediate effector function. However, these cells can be stimulated by DCs and differentiate into CCR7- effector T cells upon secondary antigenic stimulation. Effector T cells migrate mainly through peripheral tissues and their migration is mediated by integrins and chemokine receptors (Baggiolini, 1998, Sallusto and Baggiolini, 2008).

Upon antigenic stimulation, effector T cells differentiate into effector memory and central memory T cells. Both effector memory and central memory T cells differentiate from naïve T cells. Respectively, the two memory subtypes have different effectors functions; effector memory T cells have been shown to produce IL-4, IL-5 and IFN-γ, whereas central memory T cells produce IL-2 only (Sallusto et al., 1999). This highlights the fact that both memory cell subsets are required for different responses in immunity. Investigating the kinetics and diversity of all four T cell subtypes within CD4+ and CD8+ T cells populations will aid in identifying whether CBT patients form immunological memory post-transplant. Identification of these four subtypes will also aid in understanding the thymic independent reconstitution of T cells in CBT patients.

Tregs are a subpopulation of T cells that play a crucial role in regulating or suppressing other cells within the immune system, particularly through the secretion of cytokines such as IL-10, IL-35 and TGF-β (Annacker et al., 2003, Collison et al., 2007, Read et al., 2000). Tregs control the immune response to self and foreign antigens and have been proven to prevent autoimmune diseases. Tregs express surface markers such as CD4, CD25 and FoxP3 (Curiel, 2007). In HSCT recipients, the function and reconstitution of Tregs is affected and poor reconstitution of Tregs has been shown to be associated with increased incidence of cGvHD (Trenado et al., 2003, Taylor et al., 2002). In this current study, Tregs have been defined as CD3+CD4+CD25+CD127lo. Furthermore, intranuclear staining has allowed identification of Foxp3 and so Tregs have also been defined as CD3+CD4+CD25+CD127loFoxp3+. Due to their important roles, the kinetics of Treg recovery has been measured in CBT patients.

In healthy individuals, 95% of T cells in PB express the αβ TCR and the remaining 5% of T cells express the γδ TCR receptor. γδ T cells can be activated by non-MHC complex antigen presentation, predominantly via NK cell receptors and TLRs. This causes γδ T cells to have a unique role in innate and adaptive immune responses (Vantourout and Hayday, 2013). In allogeneic HSCT studies, γδ T cells increase in absolute numbers and are protective against CMV reactivation and disease (Knight et al., 2010). Herein, the percentage expression of αβ and γδ T cells was measured to understand whether there is an increase or decrease in the expression of the respective TCRs in CBT patients.

T cell activation is an important stage in T cell functionality. CD69 is known as an early marker of T cell activation and can be expressed as early as four hours after T cell activation. In turn, this promotes and initiates protein tyrosine kinases and calcium influx, which leads to the increased production of cytokines such as IL-2 and TNF-α (Santis et al., 1992). Post-antigenic challenge, T cells express CD25, which is expressed later compared to CD69. CD25 is known as a mid-level expression marker as it is expressed later compared to CD69 (Theze et al., 1996). The expression of CD25 on T cells is induced by cytokines

such as IL-2, IL-12, IL-4 and TNF-α. CD25 is the IL-2-alpha receptor chain and upon its expression on T cells it will initiate the proliferation and differentiation of T cells (Gately et al., 1991, Mitchell et al., 1989, Vassalli, 1992). HLA-DR is a class II MHC receptor and is widely expressed on APCs such as macrophages, monocytes, B cells and DCs. Subsequently, they present processed exogenous antigens to CD4+ T cells. However, T cells also express HLA-DR upon activation. HLA-DR is expressed later on T cells compared to CD69 and CD25 and is known as a late activation marker (Moriya et al., 1987). Taken together, the measurement of the respective markers have been analysed within this study to understand whether T cells are activated in CBT patients. This provides an insight into the functional activity of T cells in CBT patients.

In this chapter, the thymopoiesis of T cell recovery was assessed in CBT patients by measuring absolute counts of naïve T cells, recent thymic emigrants and genetic analysis was performed to measure TREC copy numbers within T cells. This was performed to understand and identify the kinetics of thymic dependent recovery in CBT patients. Immunophenotyping was also used to measure the absolute counts of T cell subsets in the PB of CBT patients. This was performed to measure the kinetics of thymic-independent recovery and to define the time taken for T cell reconstitution to occur in CBT patients. Furthermore, the percentage expression of αβ and γδ T cells was measured to assess the kinetics of TCR recovery in CBT patients and the expression pattern of T cell activation markers was measured to identify whether T cells are activated post-CBT.

The specific aims of this chapter are the following:

1. Map the kinetics of recovery of CD45RA+CCR7+ (CD4+ and CD8+) naïve T cells in CBT patients.
2. Map the kinetics of recovery of CD31+ recent thymic emigrants (CD4+ and CD8+) in CBT patients.
3. Map kinetics of recovery of TRECs in CBT patients.
4. Identify the ratio of CD4:CD8 T cells in CBT patients.
5. Map the kinetics and diversity of CD4+ and CD8+ effector T cells in CBT patients.
6. Map the kinetics of recovery of CD4+ and CD8+ effector memory T cells in CBT patients.
7. Map the kinetics of recovery of CD4+ and CD8+ central memory T cells in CBT patients.
8. Map the kinetics of recovery of regulatory T cells in CBT patients.
9. Determine expression patterns of αβ and γδ T cells within CBT patients.
10. Determine the expression patterns of CD25, CD69 and HLA-DR, activation markers within CD4+ and CD8+ T cells in CBT patients.

6.2 CD4+ and CD8+ T cell ratios in cord blood transplant patients

Monitoring the reconstitution patterns of CD4+ and CD8+ T cells provides an indication of thymic recovery. The absolute count measurements for CD4+ and CD8+ T cells have been shown in Chapter 3, Figure 3.4 and Figure 3.5, respectively. These results have displayed that CD8+ T cells reconstitute within CBT patients by 365 days post-transplant. However, CD4+ T cell reconstitution could take longer than 720 days to reconstitute within CBT patients.

In this current study, the ratio of CD4:CD8 T cells has been calculated to understand what ratio is seen in CBT patients, as shown in Figure 6.1. The ratios of CD4:CD8 T cells post-transplant are as follows: day +28 (80: 20), +60 (71:29), +100 (69:31), +180 (70:30), +365 (56:44) and +720 (55:45). Respectively, the CD4:CD8 T cell ratio in healthy control PB was 63:37. Overall, this data suggests that by 720 days post-transplant, CBT patients have a 1:1 ratio of CD4:CD8 T cells. Furthermore, this indicates that CBT patients have a similar ratio of CD4:CD8 T cells compared to healthy controls.

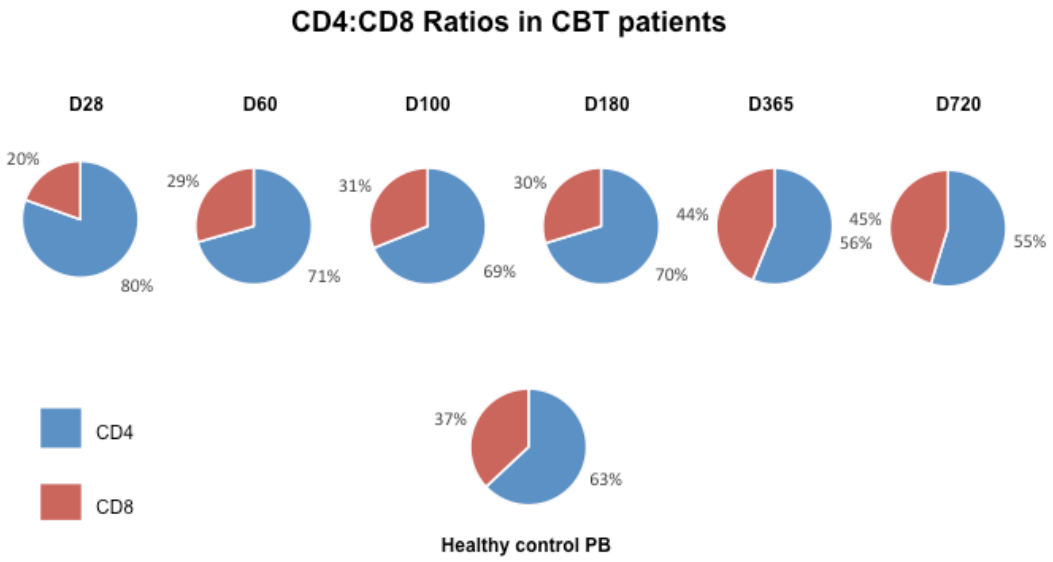


Figure 6.1 Ratio of CD4 to CD8 T cells in CBT patients. Pie charts are used to represent the ratio of CD4+ and CD8+ T cells in CBT patients. At each time point, the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26 and D720 = 14. Furthermore, n=10 healthy control samples were also tested. The data presented in the graph above are the mean averages of CD4+ and CD8+ T cells in CBT patients at the respective patient sample time points.

6.3 Naïve T cell count recovery following allogeneic cord blood transplantation

In Chapter 3, it was shown that the reconstitution of CD3+, CD4+ and CD8+ T cells is delayed. With this in mind, it was hypothesised that naïve T cell reconstitution is delayed in CBT patients. To test this hypothesis, the absolute counts of T cell subsets were identified and measured in CBT patients using flow cytometry. In this chapter, the kinetics of naïve T cell reconstitution was assessed via detection of cell surface markers such as: CD4, CD8 CD45RA and CCR7 shown in Figure 6.2 and Figure 6.3.

6.3.1 Naïve CD4+ T cell count recovery following allogeneic cord blood transplantation

At 28 days post-transplant, the median number (26 cells/μl) of naïve CD4+ T cells was significantly lower compared to healthy control PB (345 cells/μl) and the interquartile range was also narrower in CBT patients (5-69 cells/μl) compared to healthy control PB (166-658 cells/μl) (p=0.004). At 60 days post-CBT, median counts of naïve CD4+ T cells (16 cells/μl) were significantly lower compared to healthy control PB and the interquartile range was narrower (2.5-35 cells/μl) compared to healthy control PB (p=0.002). At day 100 post-CBT, median absolute counts of CD4+ naïve T cells (7 cells/μl) were at the lowest level compared to all of the respective time points and was significantly lower compared to the median healthy control PB value (p=0.002). Respectively at the same time point, the interquartile range (3.5-70.5 cells/μl) was narrower compared to healthy control PB. At 180 days post-CBT, the interquartile range (3-70 cells/μl) was narrower compared to the interquartile range observed in healthy control PB. Furthermore, the median levels in CBT patients (26 cells/μl) at 180 days were also significantly lower compared to healthy control PB (p=0.003). At 365 and 720 days post-CBT, median absolute counts in patients remained lower compared to healthy control PB. Additionally, the interquartile range was narrower at 365 (8-86 cells/μl) and 720 days post-CBT (10-138 cells/μl) compared to healthy control PB. Overall, this data suggests that the absolute value of CD4+ naïve T cells is significantly lower compared to healthy

controls. Furthermore, this data shows that the reconstitution of naïve CD4+ T cells is delayed in CBT patients and could take longer than 720 days to reconstitute in CBT patients.

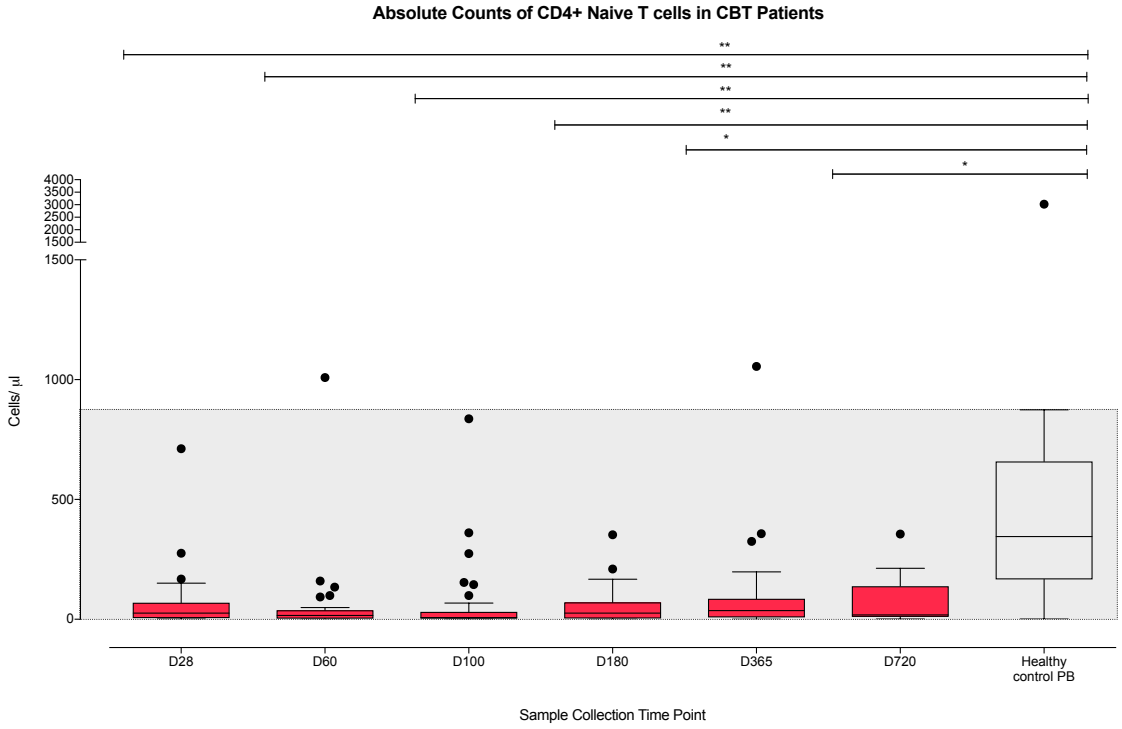


Figure 6.2 Absolute counts of CD4+ naive T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD4+ naïve T cells within CBT patients. Flow cytometry was performed to quantify CD4+ naïve T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as tukey analysis. $p<0.05$ (*) and $p<0.01$ (**).

6.3.2 Naïve CD8+ T cell count recovery following allogeneic cord blood transplantation

At 28 days post-CBT, the median absolute number of CD8+ naïve T cells (8 cells/μl) was significantly lower compared to healthy control PB (158 cells/μl) ($p= 0.005$). At successive time points between 60 days and 180 days post-CBT, the median absolute level of CD8+ naïve T cell numbers remained lower compared to healthy control PB. Respectively, the interquartile ranges between 28 days and 180 days post CBT were narrower compared to the healthy control PB (37-219 cells/μl). By 365 days post-CBT, the median absolute counts of CD8+ naïve T cells increased but remained lower compared to healthy control PB. However, the interquartile range was broadest at 365 days (3-179 cells/μl) post-CBT compared to healthy control PB. At 720 days post-CBT, the median absolute counts increased (63 cells/μl). However, this was lower compared to healthy control PB. Overall, the absolute numbers of CD8+ naïve T cells were lower in CBT recipients compared to healthy controls. This suggests that the reconstitution of CD8+ naïve T cells is delayed in CBT patients for up to 720 days post-CBT.

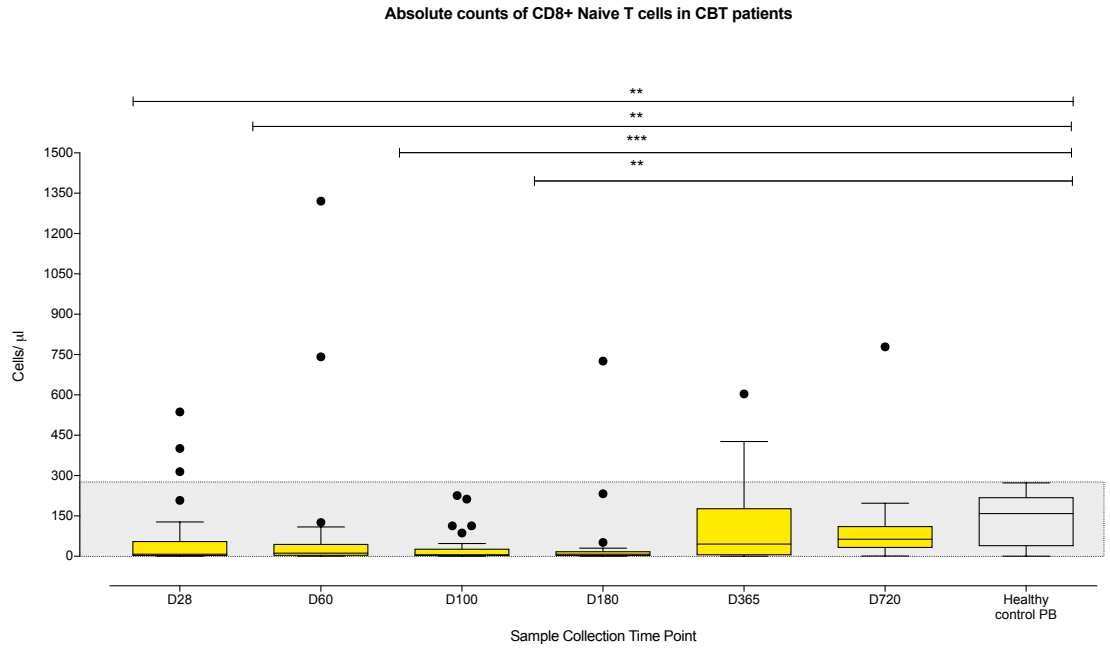


Figure 6.3 Absolute counts of CD8+ Naive T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD8+ naïve T cells within CBT patients. Flow cytometry was performed to quantify CD8+ naïve T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as tukey analysis. $p<0.01$ (**) and $p<0.001$ (***).

6.4 CD31+ recent thymic emigrant cell count recovery following allogeneic cord blood transplantation

Thymopoeisis can be measured by quantifying the absolute counts of RTEs in CBT patients. The expression of CD31 on T cells is associated with naive T cells that egress from the thymus. In this study, both CD4+ and CD8+ RTEs have been quantified to assess the kinetics of naïve T cells from the thymus of CBT patients, as shown in Figure 6.4 and Figure 6.5.

6.4.1 CD4+CD31+ recent thymic emigrant cell count recovery following allogeneic cord blood transplantation

At 28 days post-CBT, median absolute counts of CD4+ RTEs (16 cells/μl) were significantly lower compared to healthy control PB (279 cells/μl). Additionally, the interquartile range of CD4+ RTEs at 28 days post-CBT was narrower in patients compared to healthy control PB (175-358 cells/μl) (p=0.0001). At successive time points up to 720 days, the median levels of CD4+ RTEs remained low compared to healthy control PB. Respectively, the interquartile ranges of CD4+ RTEs in CBT patients were narrower compared to healthy control PB. At all time points between 60 days and 720 days, CBT recipients showed to have significantly lower absolute counts of RTEs compared to healthy control PB. Furthermore, the broadest interquartile range was seen at 365 days post-CBT. This data suggests that the number of RTEs post-CBT were considerably lower in CBT patients compared to healthy control PB and the reconstitution of RTEs could take longer than 720 days.

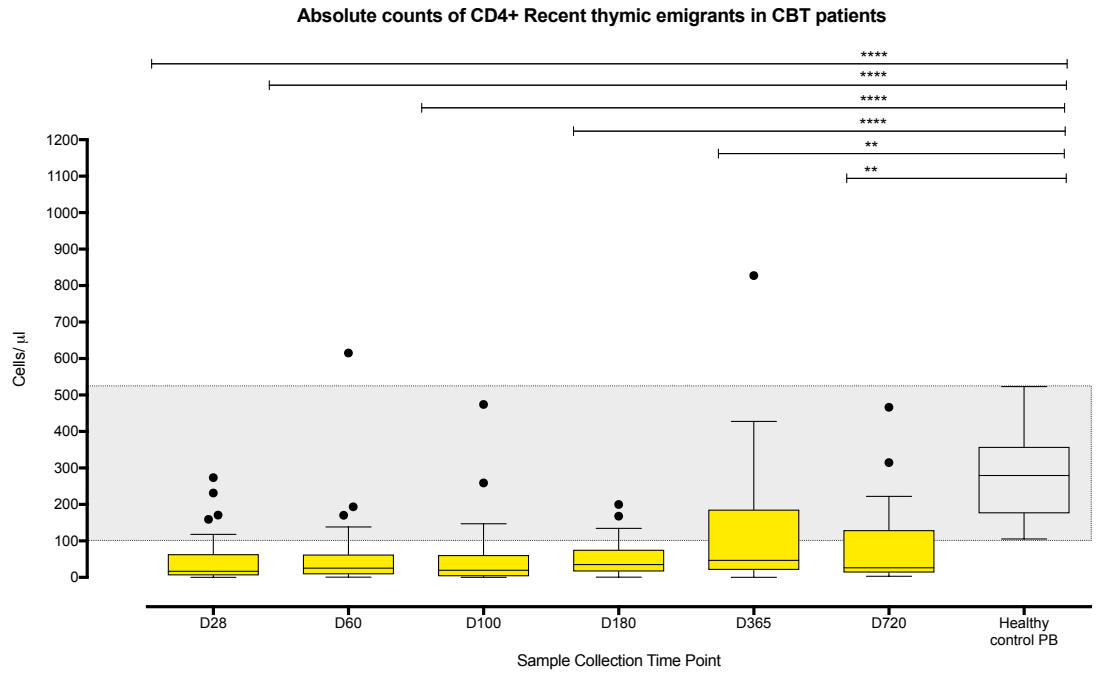


Figure 6.4 Absolute counts of CD4+ recent thymic emigrants in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD4+ RTEs within CBT patients. Flow cytometry was performed to quantify CD4+ RTEs. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as tukey analysis. $p<0.01$ (**) and $p<0.001$ (***).

6.4.2 CD8+ CD31+ recent thymic emigrant cell count recovery following allogeneic cord blood transplantation

CBT recipients at 28 days post transplant had a significantly lower median absolute count of CD8+ RTEs (2 cells/μl) compared to healthy control PB (251 cells/μl) (p=0.0001). Additionally, the interquartile range of CD8+ RTEs in CBT patients at 28 days was lower (0-3 cells/μl) compared to healthy control PB (204-310 cells/μl). The interquartile ranges also remained narrow between 60 and 100 days post-CBT and were narrower compared to healthy control PB. Median absolute counts of CD8+ RTEs were also significantly lower in CBT patients up to 180 days compared to healthy control PB. Respectively, at 180 days post-CBT the interquartile range was broader compared to all other time points (3-212 cells/μl). At successive time points between 180 and 720 days, the median absolute counts of CD8+ naïve T cells were significantly lower compared to healthy control PB. Overall, this data suggests that there were very low absolute counts of CD8+ RTEs in CBT recipients post-transplant. Furthermore, absolute counts were significantly lower compared to healthy controls. In combination with CD4+ recent thymic emigrant data, this data also suggests that reconstitution of CD8+ RTEs could take longer than 720 days post-CBT.

Figure 6.5 Absolute counts of CD8+ recent thymic emigrants in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD8+ RTEs within CBT patients. Flow cytometry was performed to quantify CD8+ RTEs. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis. $p<0.01$ (**) and $p<0.001$ (***) and $p<0.0001$ (****).

6.5 TREC quantification in cord blood transplant patients

TRECs are excision loops of DNA that are formed as a gene bi-product during T cell maturation within the thymus. Naïve T cells express high quantities of TRECs and once these T cells are released from the thymus into the periphery, the excision loops are diluted through clonal expansion of T cells. TRECs were quantified in CBT patients to assess the thymic output of naïve T cells, as shown in Figure 6.6. Absolute copy numbers of TRECs were quantified as described in Chapter 2, Section 2.7.20. Throughout the six time points in this current study, TREC copy numbers were not detected in the majority of patients. However, in ten patients TRECs were quantified at different time points throughout the study and these patients had higher levels of TRECs compared to healthy controls. Furthermore, the ten TREC positive samples were CD31+CD45RA+. However, the TREC negative samples were CD31+CD45RA+. Overall, this suggests that thymic output is delayed in CBT patients and could take longer than 720 days to improve.

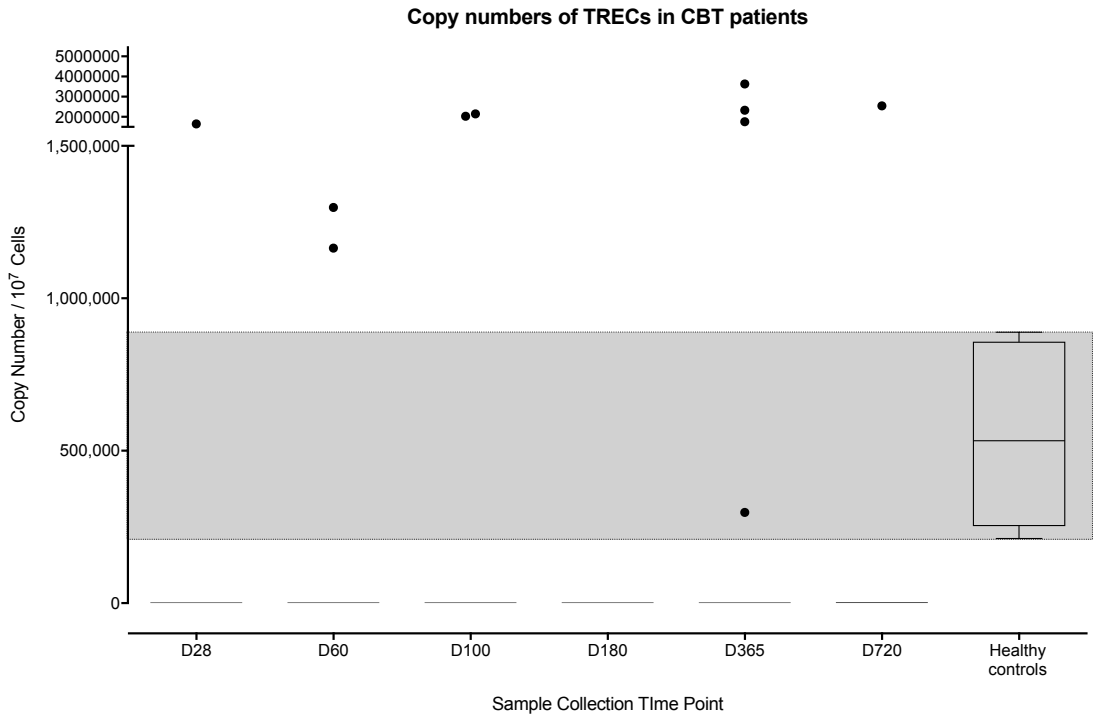


Figure 6.6 Copy number of TRECs in CBT patients. Box and whisker plots showing the copy number of TREC genes, copy number per 10^7 Cells. A real-time PCR assay was performed to quantify TREC genes. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy controls = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis.

6.6 Effector T cell count recovery following allogeneic cord blood transplantation

Upon antigenic stimulation, naïve T cells differentiate into effector T cells. In this current study, CD4+ and CD8+ effector T cells are defined as $CD3^+CD4^+CD45RA^+CCR7^-$ and $CD3^+CD8^+CD45RA^+CCR7^-$ T cells, respectively (Figure 6.7 and Figure 6.8). The quantification of effector T cells has been performed to understand the kinetics of reconstitution of these cells in CBT patients. This T cell subset is a crucial development stage that could lead to further maturation and differentiation into central memory and effector memory T cells.

6.6.1 CD4+ effector T cell count recovery following allogeneic cord blood transplantation

At 28 days post-CBT, the median absolute counts (3 cells/μl) of CD4+ effector T cells were significantly lower compared to healthy control PB (26.5 cells/μl) ($p=0.02$). Furthermore, the interquartile range was narrower (0-19 cells/μl) in CBT patients compared to healthy control PB (8-64 cells/μl). Between 60 and 100 days post-CBT, the median absolute counts increased and the interquartile range (1-53 cells/μl) at 100 days was equivalent to the interquartile range of healthy control PB (8-64 cells/μl). By 180 days post-CBT, median levels of CD4+ effector T cells (25 cells/μl) were similar to that seen in healthy control PB (26 cells/μl). Furthermore, the interquartile range within CBT patients (3-102 cells/μl) at day 180 was broader compared to healthy control PB. By 365 days post-CBT, median absolute counts of CD4+ effector T cells increased to 32 cells/μl. The interquartile range also broadened (4-145 cells/μl) within CBT patients and was broader compared to healthy control PB. However, at 720 days, median levels of CD4+ effector T cells decreased (14 cells/μl) and the interquartile range (0-54 cells/μl) was narrower compared to healthy control PB. Overall, this data suggests that the reconstitution of CD4+ effector T cells occurs by 60 days as the median absolute counts are equivalent to healthy

adults. Furthermore, the median absolute number of CD4+ effector T cells reaches the healthy control range over the two-year follow up.

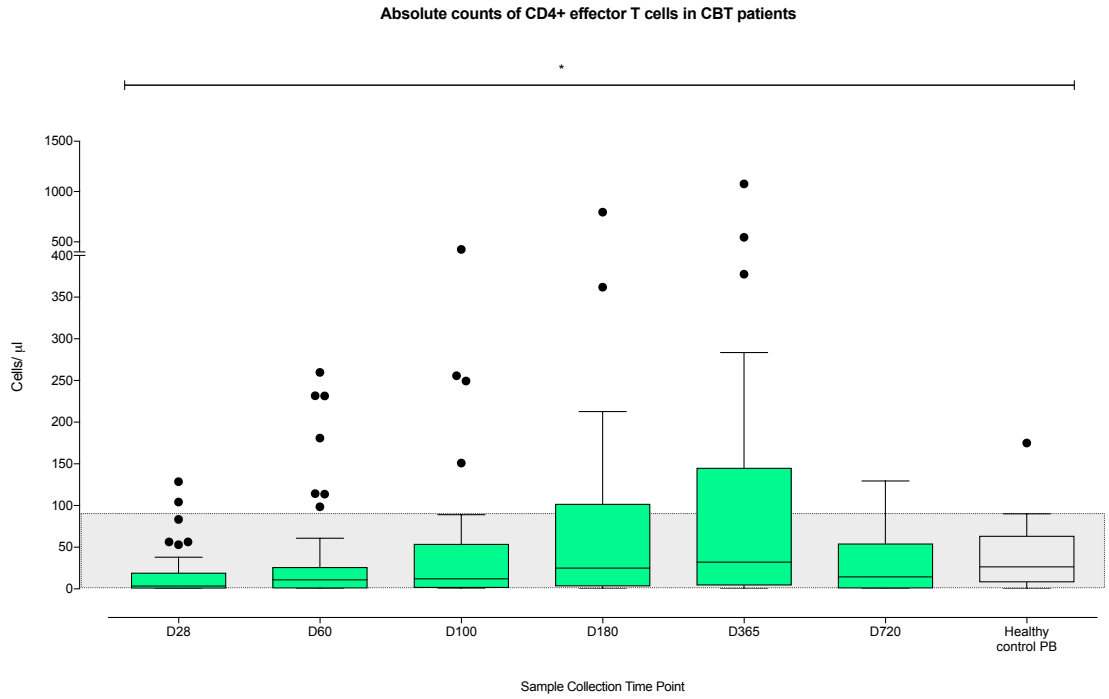


Figure 6.7 Absolute counts of CD4+ effector T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD4+ effector T cells within CBT patients Flow cytometry was performed to quantify CD4+ effector T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis. $p<0.05$ (*).

6.6.2 CD8+ effector T cell count recovery following allogeneic cord blood transplantation

At 28 days post-CBT, patients had a significantly lower median absolute count of CD8+ effector T cells (2 cells/μl) compared to healthy control PB (146 cells/μl) ($p=0.006$). Respectively, at 28 days post-CBT, the interquartile range of CD8+ effector T cells (0-18 cells/μl) was narrower compared to healthy control PB (19-223 cells/μl). The median absolute count of CD8+ effector T cells remained low at subsequent time points up to 720 days post-CBT compared to healthy control PB. The broadest interquartile range was recorded at 180 days post-CBT (0-365 cells/μl) and was broader compared to healthy control PB (19-223 cells/μl). Overall, this suggests that CBT patients had low absolute numbers of CD8+ effector T cells post-transplant and the absolute counts of CD8+ effector T cells remained low for up to 720 days post-CBT.

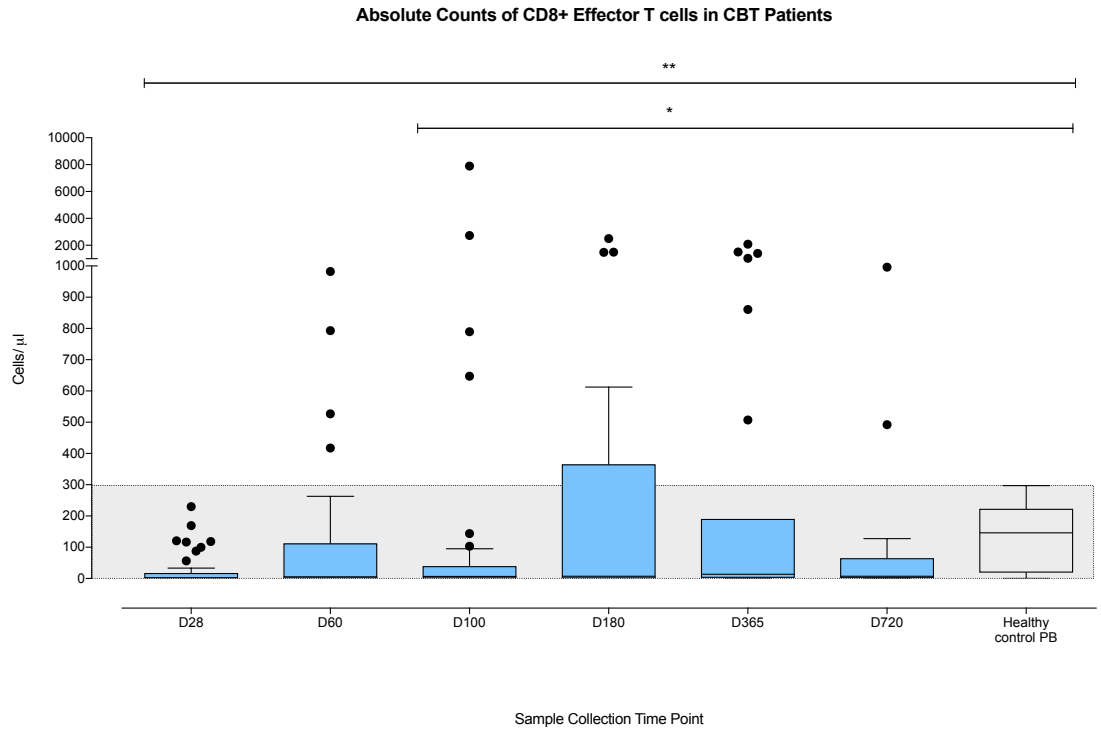


Figure 6.8 Absolute count of CD8+ effector T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD8+ effector T cells within CBT patients. Flow cytometry was performed to quantify CD8+ effector T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis, $p<0.05$ (*) and $p<0.01$ (**).

6.7 Effector memory T cell count recovery following allogeneic cord blood transplantation

Effector memory T cells, also known as CCR7- T cells, migrate to sites of inflammation and display immediate effector functions. In CBT patients, this could include various inflammatory responses involved in GvHD or clearance of infection. Therefore, in this study the kinetics of reconstitution of CD4+ and CD8+ effector memory T cells has been measured in CBT patients, as shown in Figure 6.9 and Figure 6.10.

6.7.1 CD4+ effector memory T cell count recovery following allogeneic cord blood transplantation

At 28 days post-CBT, the median absolute count of CD4+ effector memory T cells (24 cells/μl) was significantly lower compared to healthy control PB (315 cells/μl) (p=0.0001). Furthermore, the interquartile range at 28 days post-CBT was narrower (12-46 cells/μl) compared to healthy control PB (267-392 cells/μl). At 60 days post-CBT, the median absolute count of CD4+ effector memory T cells increased in CBT patients (93 cells/μl). However, the value was significantly lower compared to healthy control PB (p=0.001). The interquartile range (50-222 cells/μl) was broader at 60 days post-CBT compared to healthy control PB. A further increase in median absolute count of CD4+ effector memory T cells was seen at 100 days post-CBT. However, the median absolute count remained below the healthy control PB median count (p=0.0136). Similarly, the interquartile range in CBT patients (43-354 cells/μl) at day 100 was broader compared to healthy control PB. The median absolute count of CD4+ effector memory T cells increased at 180 days post-CBT in patients (232 cells/μl). However, at this time point, the median absolute count of CD4+ effector memory T cells was lower compared to healthy control PB. The interquartile range at 180 days post-CBT was higher compared to healthy control PB. The median CD4+ effector memory T cell value (395 cells/μl) was the highest at 365 days post-CBT and was higher compared to healthy control PB. Respectively, at 365 days the interquartile range was broader (102-508

cells/µl) compared to healthy control PB. By 720 days post-CBT, the median absolute count of CD4+ effector memory T cells decreased (219 cells/µl). However, at this time point, the interquartile range (14-639 cells/µl) was broadest compared to all other time points and compared to healthy control PB. Overall, this data demonstrates that there is an increase in CD4+ effector memory T cells between 28 and 365 days post-CBT. Furthermore, reconstitution takes place by 365 days post-transplant.

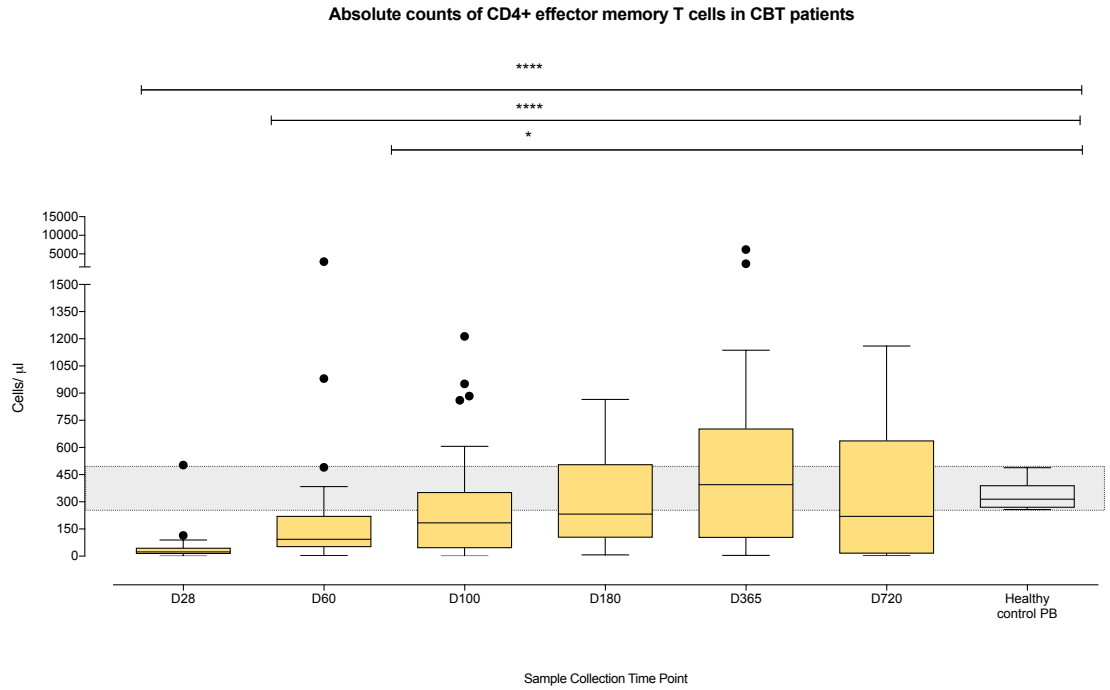


Figure 6.9 Absolute counts of CD4+ of effector memory T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD4+ effector memory T cells within CBT patients. Flow cytometry was performed to quantify CD4+ effector memory T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis, $p<0.05$ (*) and $p<0.0001$ (****).

6.7.2 CD8+ effector memory T cell count recovery following allogeneic cord blood transplantation

The median absolute count of CD8+ effector memory T cells (29 cells/µl) was significantly lower compared to healthy control PB (261 cells/µl) at 28 days post-CBT (p=0.0001). Respectively at 28 days post-CBT, the interquartile range (7-69 cells/µl) was narrower compared to successive time points and healthy control PB (93-284 cells/µl). At successive time points, there was an increase in the median absolute counts of CD8+ effector memory T cells, where the highest median count was recorded at 365 days post-CBT (384 cells/µl). This was higher compared to healthy control PB. The interquartile range also broadened between 100 days and 720 days post-CBT and respectively at these four time points the interquartile ranges recorded in patients were broader compared to healthy control PB. The broadest interquartile range was recorded at 720 days post-CBT (38-1094 cells/µl). Overall, this data shows that there is an increase in the number of CD8+ effector memory T cells between 28 and 365 days post-CBT. Furthermore, reconstitution of CD8+ effector memory T cells takes place by 100 days post-CBT.

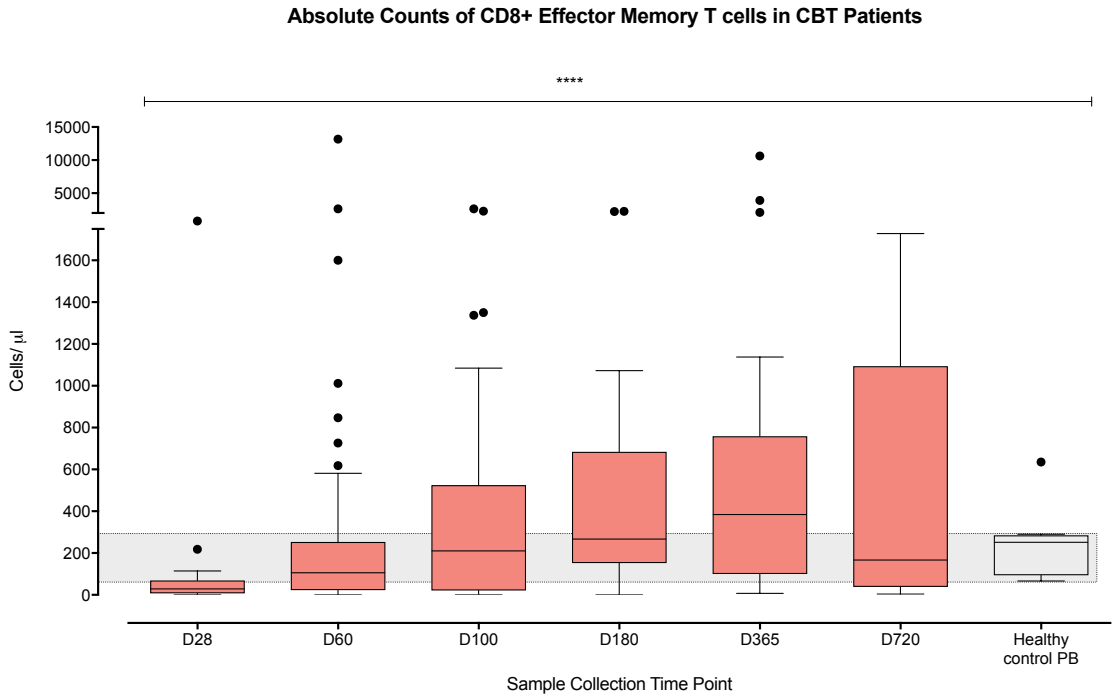


Figure 6.10 Absolute counts of CD8+ effector memory T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD8+ effector memory T cells within CBT patients. Flow cytometry was performed to quantify CD8+ effector memory T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis $p<0.0001$ (****).

6.8 Central memory T cell count recovery following allogeneic cord blood transplantation

CCR7+ central memory T cells are known to express lymph node homing receptors. However, they lack immediate effector functions. Once central memory T cells migrate to the lymph nodes or are stimulated by antigens, they differentiate into CCR7- effector memory T cells. Furthermore, they could play a role in GvHD mediated responses, clearance of infection and could be involved in GvL responses. With this in mind, the kinetics of central memory T cell reconstitution has been measured in CBT patients, as shown in Figure 6.11 and Figure 6.12.

6.8.1 CD4+ central memory T cell count recovery following allogeneic cord blood transplantation

At 28 days post-CBT, the absolute median absolute counts of central memory T cells (6 cells/μl) were lower compared to healthy control PB (407 cells/μl) (p=0.0001). Furthermore, the median value was lowest at 28 days post CBT compared to all of the other time points. Respectively, the interquartile range (1-27 cells/μl) at 28 days was narrower compared to all other time points and the healthy control PB interquartile range (318-682 cells/μl.) The median absolute counts of CD4+ central memory T cells were significantly lower compared to healthy control PB for the successive time points (+60 days = 6 cells/μl, +100 days = 4 cells/μl, +180 days = 48 cells/μl, +365 days = 83 cells/μl and +720 days = 245 cells/μl (p=0.0001, p=0.0001, p=0.0001, p=0.001 and p=0.009, respectively)). The interquartile ranges were broader at these respective time points compared to 28 days post-CBT. However, the interquartile ranges were narrower between 60 and 720 days compared to healthy control PB. The broadest interquartile range was recorded at 720 days in CBT patients (95-341 cells/μl). Overall, this data shows that the absolute median count of CD4+ central memory T cells were significantly lower in CBT patients compared to healthy control PB. Therefore, this suggests that reconstitution of CD4+ central memory T cells could take longer than 720 days in CBT patients.

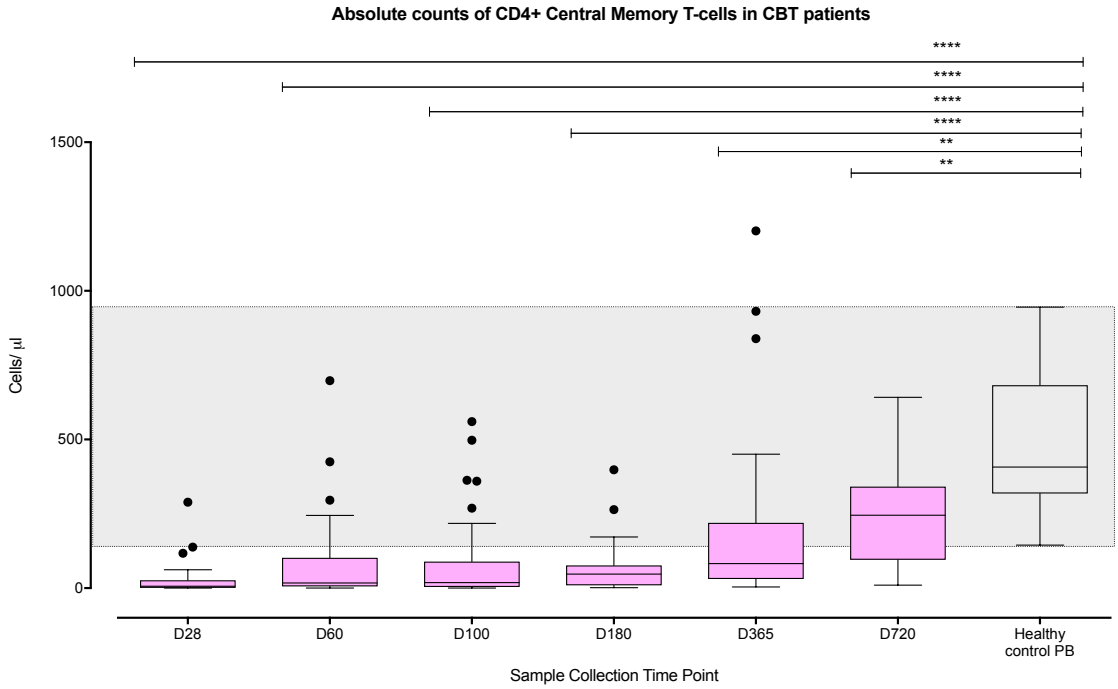


Figure 6.11 Absolute counts of CD4+ central memory T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD4+ central memory T cells within CBT patients. Flow cytometry was performed to quantify CD4+ central memory T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis, $p<0.01$ (**) and $p<0.0001$ (****).

6.8.2 CD8+ central memory T cell count recovery following allogeneic cord blood transplantation

Median absolute levels of CD8+ central memory T cells were significantly lower in CBT patients (3 cells/μl) at 28 days post-transplant compared to healthy control PB (21 cells/μl) (p=0.0003). Respectively, the interquartile range in CBT patients at 28 days (0-11 cells/μl) was narrower compared to healthy control PB (15-181 cells/μl). The median absolute counts of CD8+ central memory T cells increased at subsequent time points. The highest median absolute counts of CD8+ central memory T cells were measured in CBT patients at 365 (87 cells/μl) and 720 days (221 cells/μl) post-transplant. At 720 days, the median absolute count of CD8+ central memory T cells was higher compared to healthy control PB. The broadest interquartile ranges were recorded at 365 days (9-423 cells/μl) and 720 days (42-462 cells/μl) post-CBT, compared to healthy control PB. This data suggests that the number of CD8+ central memory T cells increased over the 720 day follow up period in CBT patients. The greatest range of cells was recorded in patients between 365 and 720 days post-transplant. This therefore indicates that CD8+ central memory T cells reconstitute by 365 days post-transplant.

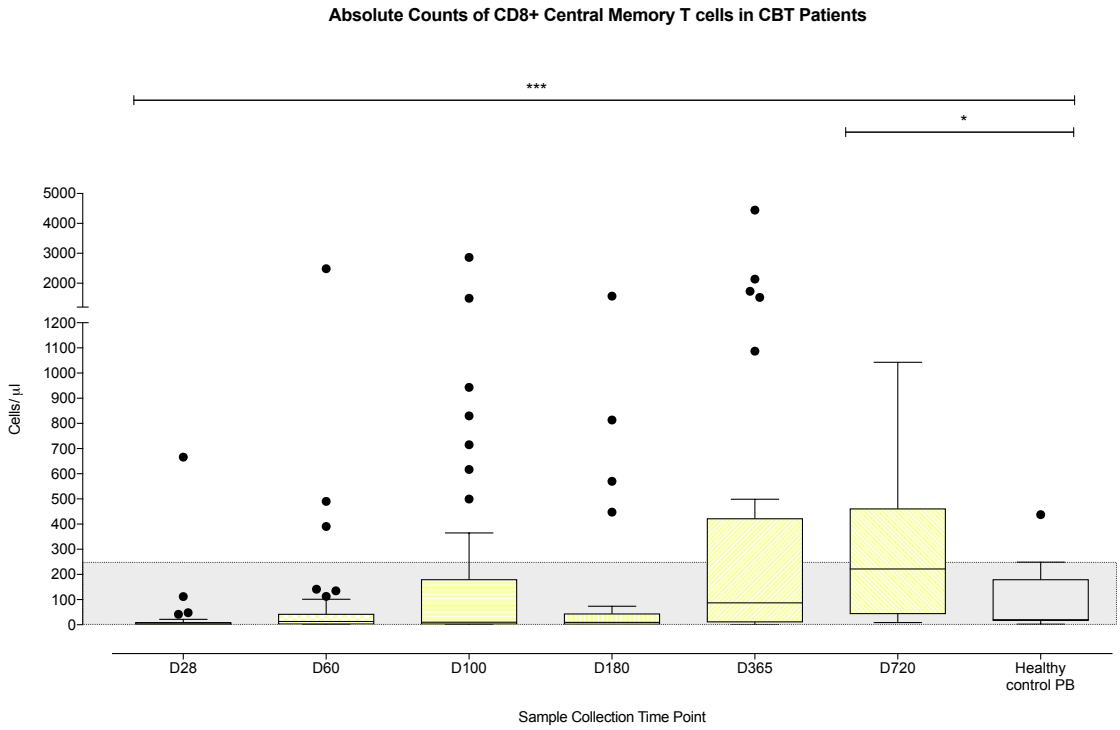


Figure 6.12 Absolute count of CD8+ central memory T cells in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of CD8+ central memory T cells within CBT patients. Flow cytometry was performed to quantify CD8+ central memory T cells. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis, $p<0.05$ (*) and $p<0.001$ (***).

6.9 CD4+ and CD8+ T cell stages in cord blood transplant patients

In this study, the composition of the CD4+ T cell and CD8+ T cell compartment was assessed to better understand the kinetics and diversity of CD4+ and CD8+ T cells in CBT patients, as shown in Figure 6.13 and Figure 6.14. Understanding the diversity of recovering subsets within CD4+ and CD8+ T cell populations will provide an insight into the reconstitution patterns of different T cell subsets within the respective T cell compartments in CBT patients.

6.9.1 T stages within the CD4+ T cell population of cord blood transplant patients

At 28 days post-CBT, patients had a high proportion of naïve T cells at 43% in the CD4+ T cell population. However, at successive time-points the percentage of naïve T cells decreased to 4% by day 720, which was lower compared to healthy control PB (34%). There were an increase in the proportion of CD4+ effector T cells between 28 days and 100 days post-CBT, from 40% to 82%. Throughout the first three time points, the percentage of CD4+ effector T cells were higher compared to healthy control PB (3%). However, the percentages of CD4+ effector T cells between 100 and 720 days post-CBT were similar to healthy control PB.

At 28 days post-CBT, 40% of CD4 T cells were CD4+ effector memory T cells. Through subsequent time-points, the percentage of CD4+ effector memory T cells increased to 71% by day 180. However, between day 180 and 720 there was a decrease from 71% to 44%. When compared to healthy control PB (27%), CBT patients had a higher percentage of CD4+ effector memory T cells. At 28 days post-CBT, 11% of the CD4+ T cell compartment consisted of CD4+ central memory T cells. There was no change in this percentage for up to 365 days. However, at 720 days post-CBT, 49% of CD4+ T cells were CD4+ central memory T cells, which were higher compared to healthy control PB (36%). Overall, our data suggests that the naïve T cell population decreased over two years and there was an increase in the effector memory and central memory population within the CD4+ T cell population. By 720 days post-transplant it can

be seen that there was a higher percentage of central memory and effector memory T cells within the CD4+ T cell population compared to healthy controls.

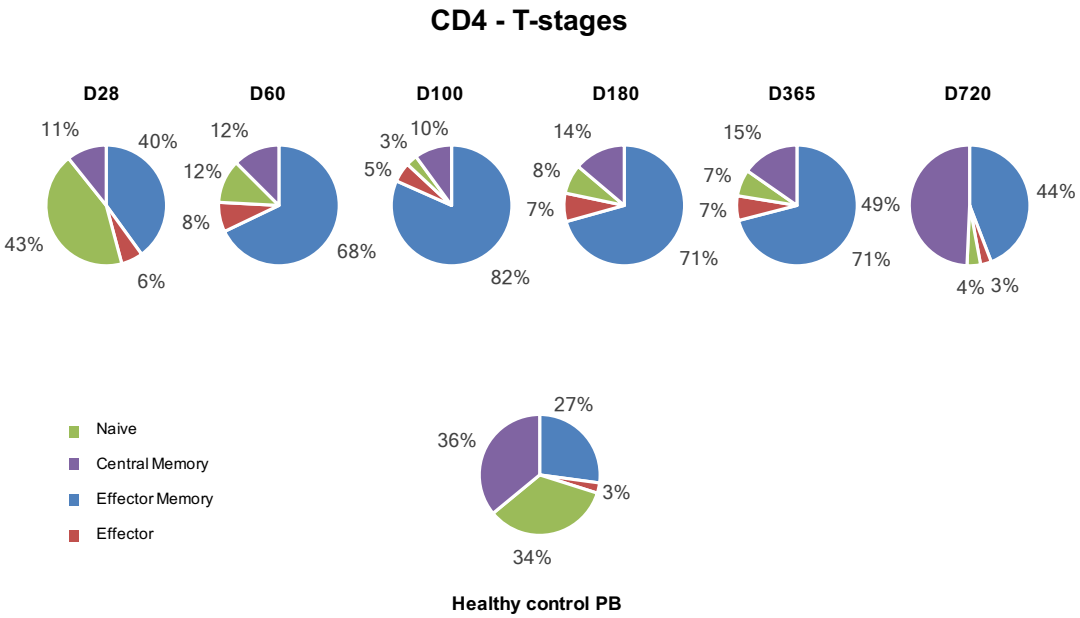


Figure 6.13 Percentage of T cell subsets within the CD4+ T cell population. Pie charts are used to represent the percentage of T cell stages in the CD4+ T cell population of CBT patients in the UK. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. The data presented in the graph above are the mean averages of the respective subpopulations of CD4+ T cells within CBT patients at the respective time points.

6.9.2 T stages within the CD8+ T cell population of cord blood transplant patients

Similar to the CD4+ T cell compartment, the percentages of T cells within the CD8+ T cell compartment were determined. At 28 days post-CBT, 7% of CD8+ T cells were naïve. Between 60 and 180 days post-transplant, the percentage of CD8+ naïve T cells decreased down to 3%. However, at 365 days and 720 days, there was an increase in the naïve subset of CD8+ T cells (17% and 48%, respectively). By 720 days post-CBT, the percentage of naive T cells within the CD8+ T cell population was higher compared to healthy control PB.

The central memory population at 28 days was 20% of the CD8+ T cell population. At successive time points, there was a decrease in the central memory T cell population, down to 2% by 100 days post-CBT. Subsequently, the central memory population increased to 14% by 720 days post-CBT within the CD8+ T cell compartment. This was lower compared to healthy control PB (27%).

The effector memory population within the CD8+ T cell compartment was the predominating subpopulation throughout the six time points. At 28 days, 68% of CD8+ T cells were effector memory T cells. This subpopulation increased to 93% by 180 days post-CBT. This was the highest-level recorded at all six time points. The effector memory population decreased from 93% at day 180 to 36% at 720 days post-CBT. By 720 days, the percentage of effector memory T cells within patients was equivalent to the percentage within healthy control PB (37%).

The effector T cell population was a small subpopulation within the CD8+ T cell compartment. At 28 days post-CBT, 5% of CD8+ T cells were effector T cells. At subsequent time points, there was very little change in the percentage of the effector T cell population. By 720 days post-CBT, 2% of the CD8 T cells were effector T cells, which was lower compared to the same population identified within healthy control PB (23%).

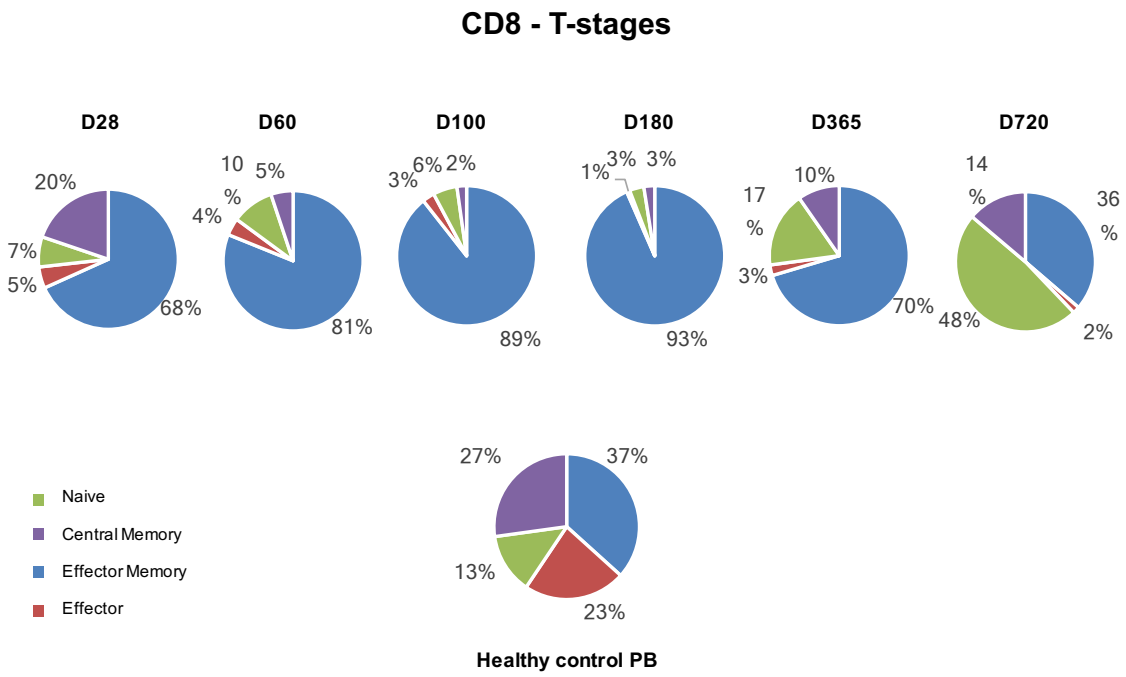


Figure 6.14 Percentage of T cell subsets within the CD8+ T cell population in CBT patients. Pie charts are used to represent the percentage of T cell stages in the CD8+ T cell population of CBT patients in the UK. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy control PB = 10. The data presented in the graph above are the mean averages of the respective subpopulations of CD8+ T cells within CBT patients at the respective time points.

6.10 Regulatory T cell count recovery following allogeneic cord blood transplant

Tregs play a crucial role in regulating immune responses and are suppressors of autoreactive and alloreactive immune responses. Due to these fundamental roles, the kinetics of Treg recovery has been measured in CBT patients, as shown in Figure 6.15.

At 28 days post-CBT median absolute counts (7 cells/μl) of Tregs were lower compared healthy control PB (54 cells/μl). Respectively, the interquartile range at 28 days (2-28 cells/μl) was narrower compared to healthy control PB (47-92 cells/μl). Subsequently, at 60 days post-CBT, there was an increase in the interquartile range (6-89 cells/μl) in CBT patients. However, median absolute counts remained significantly lower in CBT patients (12 cells/μl) compared to healthy control PB ($p=0.03$). At successive time points, there was an increase in the absolute median counts of Tregs within patients between 100 and 720 days post-CBT. The interquartile range was broadest at 365 days post-CBT (20-516 cells/μl) and was broader compared to healthy control PB. However, by 720 days post-CBT, the median absolute count of Tregs in CBT patients (26 cells/μl) was significantly lower compared to healthy control PB (54 cells/μl) ($p=0.04$). Overall, this data shows that Tregs reconstitute in CBT patients by 365 days post-transplant.

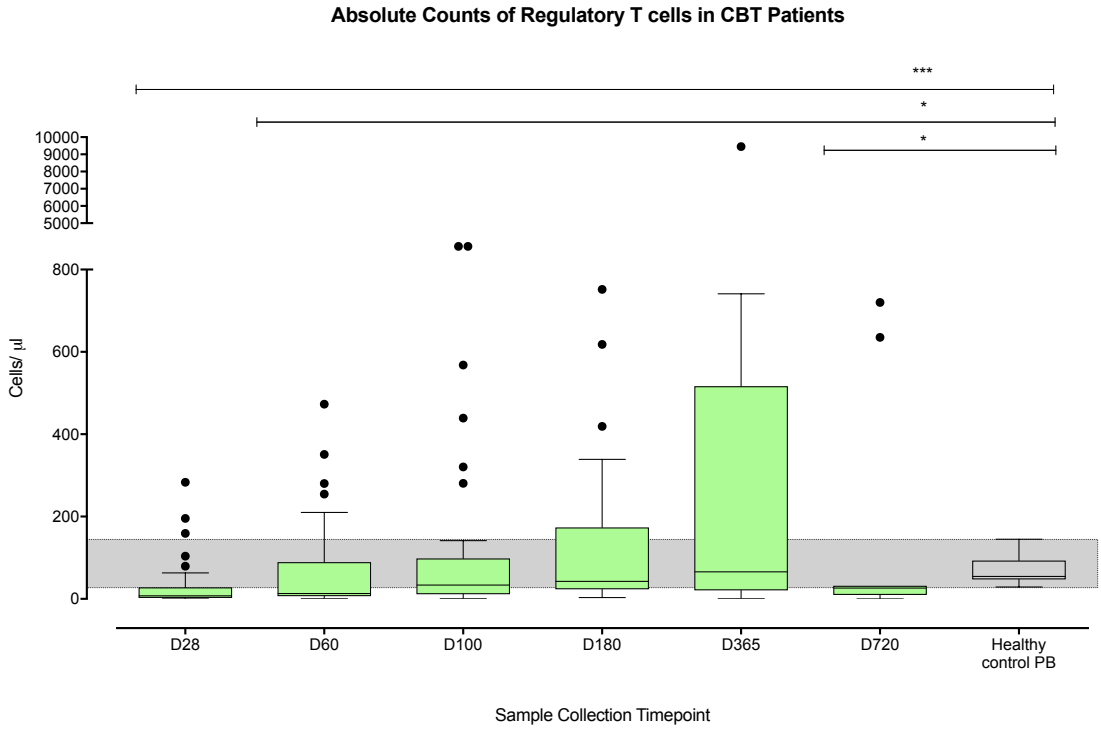


Figure 6.15 Absolute counts of Tregs in CBT patients. Box and whisker plots showing the absolute counts, cells per microliter, of Tregs within CBT patients, in the UK. Flow cytometry was performed to quantify Tregs. At each time point the following number of samples were acquired: D28 = 44, D60 = 42, D100 = 25, D180 = 26, D365 = 26, D720 = 14 and healthy adult PB = 10. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis, $p<0.05$ (*) and $p<0.001$ (***).

6.11 αβ and γδ T cells within cord blood transplant patients

In healthy individuals, 95% of the T cells within PB express the αβ TCR and the remaining express the γδ TCR (Vantourout and Hayday, 2013). However, the percentage or number of αβ and γδ T cells that are present within CBT patients is not well documented. The percentages of the respective TCRs were measured in CBT patients, as shown in Figure 6.16 A-D and Figure 6.17. This was performed on thawed PBMCs from CBT patients and healthy controls.

There were a higher percentage of αβ T cells within the CD4+ and CD8+ T cell population (figure 6.16A and 6.16B) compared to γδ T cells (figure 6.16C and 6.17D). At 28 days post CBT, the median percentage levels of αβ CD4+ T cells were equivalent to levels seen in healthy controls. However, median percentage levels decreased between 60 and 100 days were lower compared to healthy controls. The median levels of CD8+ αβ T cells (figure 6.16B) were equivalent to healthy controls. Between 28 and 100 days post-CBT, the median levels of CD8+ αβ T cells decreased and did not fall below 80%.

The percentage of γδ T cells was low throughout the first three time points (figure 6.16C and D). Percentage levels of both CD4+ and CD8+ γδ T cells remained below 2% in CBT patients between 28 and 100 days post-transplant. The interquartile range was equivalent to levels seen in healthy controls.

At 28, 60 and 100 days post CBT patients had significantly lower expression levels of TCR αβ in CD3+CD4-CD8- T cells (Figure 6.17) compared to healthy controls (D60 $p=0.01$ and D100 $p=0.01$). Respectively, at 28, 60 and 100 days post transplant CBT patients had significantly lower expression of TCR γδ expression in CD3+CD4-CD8- T cells compared to healthy controls (D28, D60 and D100 $p=0.001$).

Overall, this data suggests that the percentage of αβ and γδ T cells within CBT patients post-transplant is within the same range observed in healthy controls. However, the expression of TCR αβ and γδ were significantly lower in CBT patients compared to healthy controls within CD3+CD4$^-$CD8$^-$ T cells.

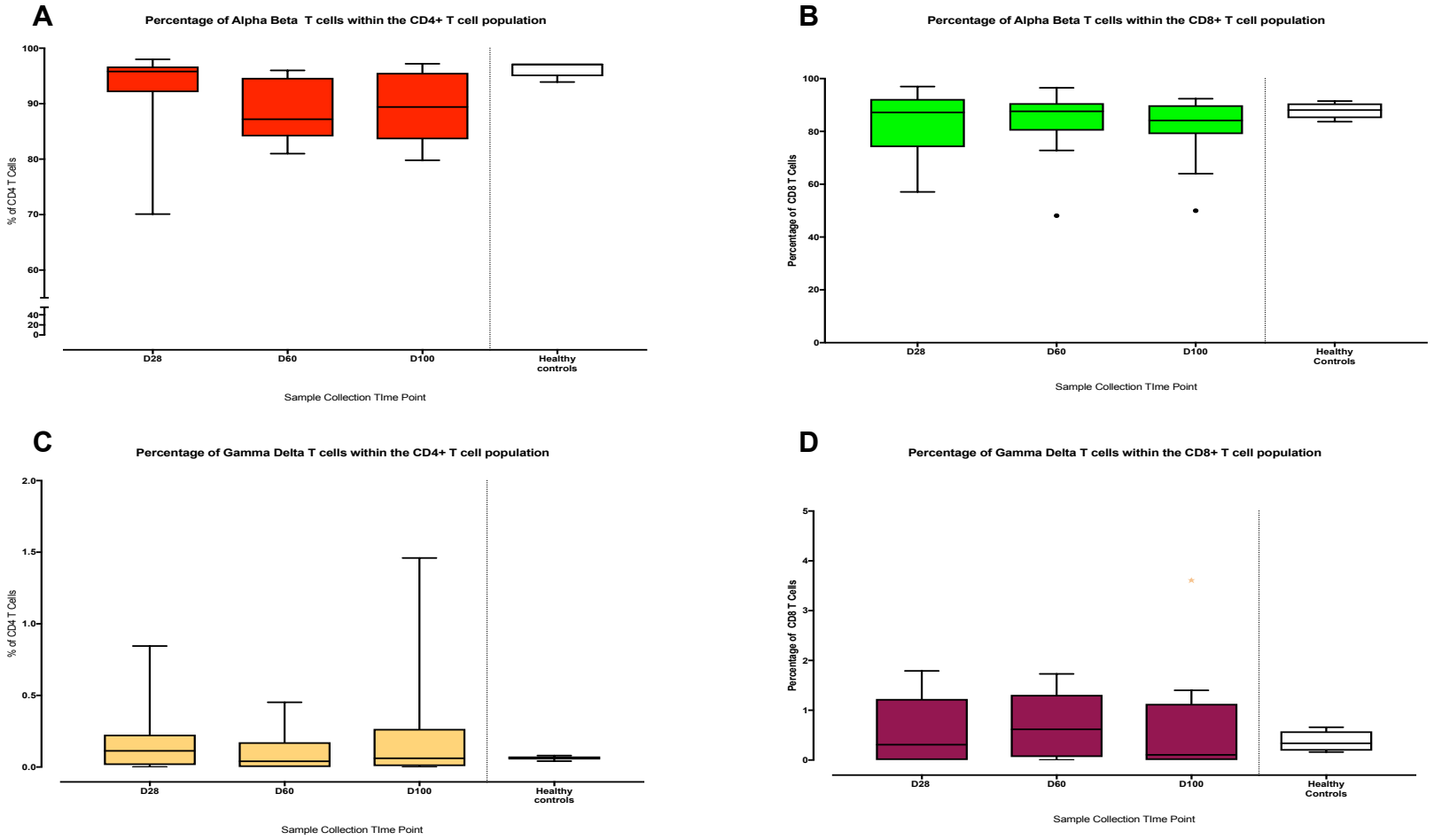


Figure 6.16 Percentage of αβ and γδ T cells in the CD4+ and CD8+ T cell population within CBT patients. Box and whisker plots showing the percentage of CD4+ αβ T cells (A), CD8+ αβ T cells (B), CD4+ γδ T cells (C), CD8+ γδ T cells (D). At each time point the following number of samples were acquired: D28 = 14, D60 = 10, D100 = 10 and healthy controls = 5. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test (no significance was observed). Results are presented as a tukey analysis.

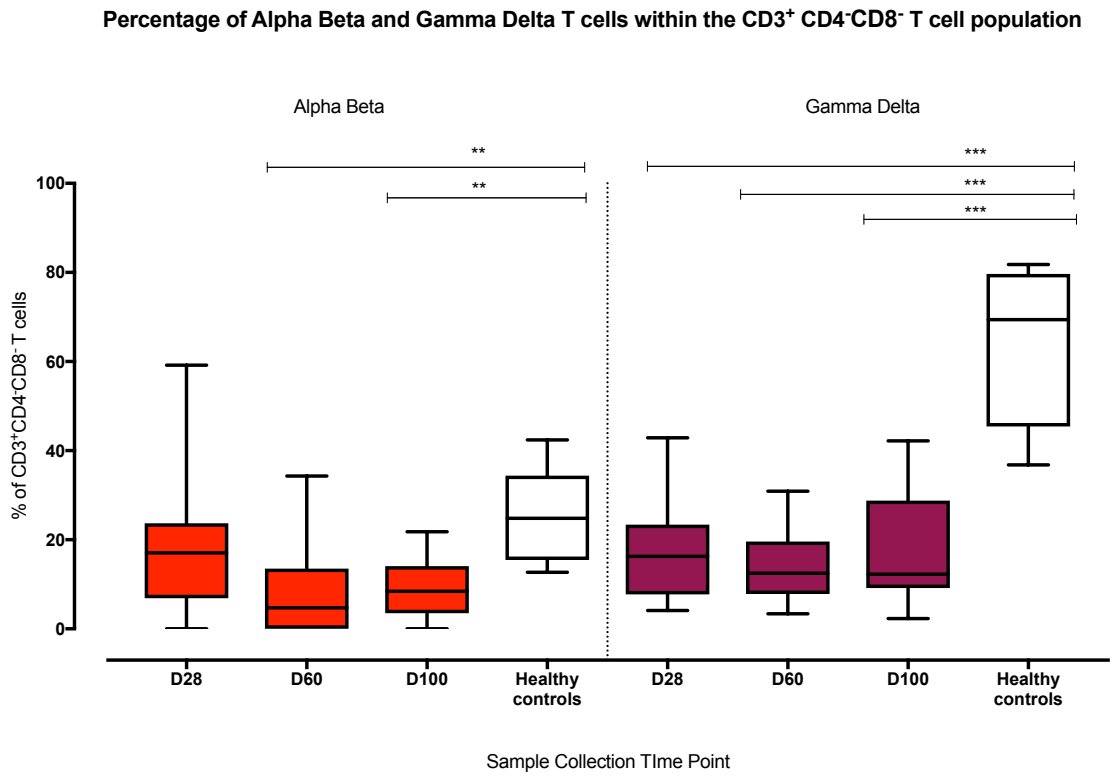


Figure 6.17 Percentage expression of TCR αβ and TCR γδ in CD3+CD4-CD8- T cells within CBT patients. Box and whisker plots showing the percentage expression TCR αβ and TCR γδ in CD3+CD4-CD8- T cells. At each time point the following number of samples were acquired: D28 = 14, D60 = 10, D100 = 10 and healthy controls = 5. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis, $p<0.01$ (**) and $p<0.001$ (***).

6.12 Percentage expression of activation markers in T cells of cord blood transplant patients

Activation molecules are expressed on T cells when antigens, mitogens and cytokines stimulate them. Early post-transplant, recovering T cell subsets that are adoptively transferred from the graft will predominantly be naïve. Upon stimulation, T cells express early, mid and late activation markers and this primes the T cells to proliferate and differentiate into effector and memory T cells. In this current study, the measurement of CD69, CD25 and HLA-DR on CD4+ and CD8+ T cells was measured (as shown in Figure 6.18 and 6.19) to understand the activation of reconstituting T cells in CBT patients. This was performed on thawed PBMCs from CBT patients and healthy controls.

6.12.1 Percentage expression of activation markers within CD4+ T cells

The median percentage of CD4+ T cells expressing CD25 (Figure 6.18A) was significantly lower at day +28 (0%, range: 0-51%) (p=0.03) post-CBT compared to healthy controls (13%, range: 10-18%). The median percentage of CD4+ T cells expressing CD25 was higher at day +60 (18%, range: 7-68%) and +100 (23%, range: 5-32%) post-CBT compared to healthy controls.

Additionally, the median percentage of CD4+ T cells expressing CD69 (Figure 6.18B) was significantly higher at day +28 (100%, range: 86-100%) and +60 (92%, range: 31-97%) (p=0.0002 and p=0.04, respectively) post-CBT compared to healthy controls (48%, range: 45-51%). The median percentage of CD4+ T cells expressing CD69 was higher at day +100 (81%, range: 39-97%) post-CBT compared to healthy controls.

Furthermore, the median percentage of CD4+ T cells expressing HLA-DR (Figure 6.18C) was significantly higher at day +28 (56%, range: 44-99%), +60 (80%, range: 47-95 %) and +100 (77%, range: 56-94%) (p=0.0007, p=0.001 and p=0.0007 respectively) post-CBT compared to healthy controls (43%, range: 38-47%).

Overall, this data suggests that there is an increase in the percentage of CD4+ T cells expressing CD25 and HLA-DR. However, there is a decrease in the percentage of CD4+ T cells expressing CD69. Furthermore, this demonstrated that CD4+ T cells in CBT patients are activated within the first three months post-CBT.

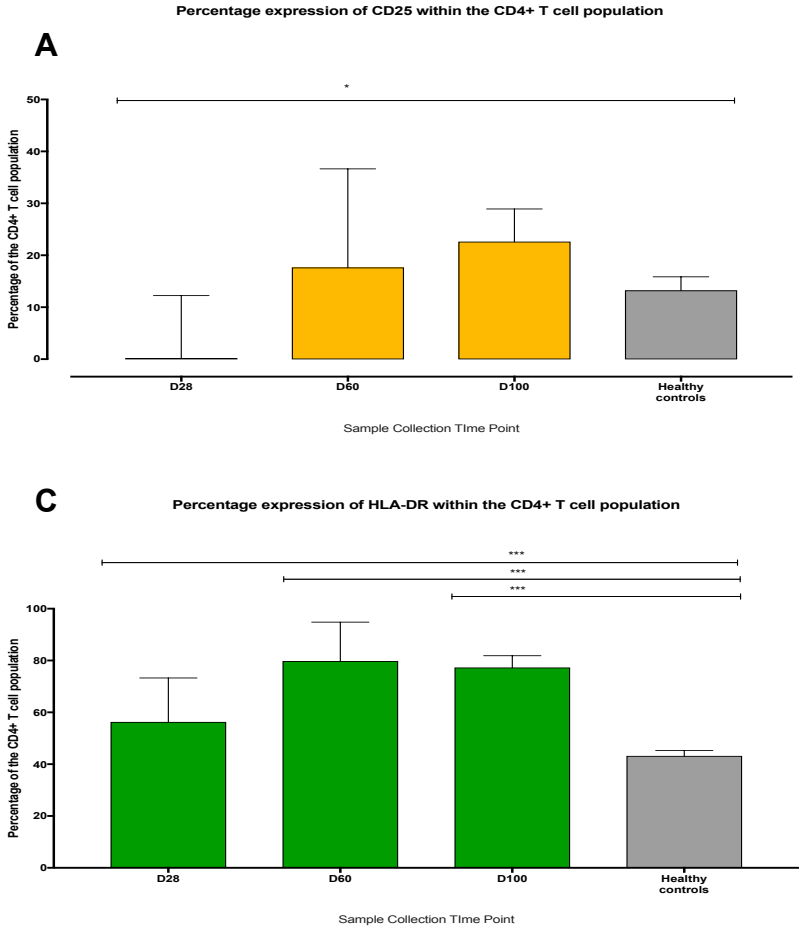


Figure 6.18 Percentage expression of activation markers (CD25, CD69 and HLA-DR) within the CD4+ T cell population of CBT patients. Box and whisker plots showing the percentage of CD25 (A), CD69 (B) and HLA-DR (C) activation markers within CD4+ T cells of CBT patients. At each time point the following number of samples were acquired: D28 = 14, D60 = 10, D100 = 10 and healthy controls = 5. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis. $p<0.05$ (*) and $p<0.001$ (***).

6.12.2 Percentage expression of activation markers within CD8+ T cells

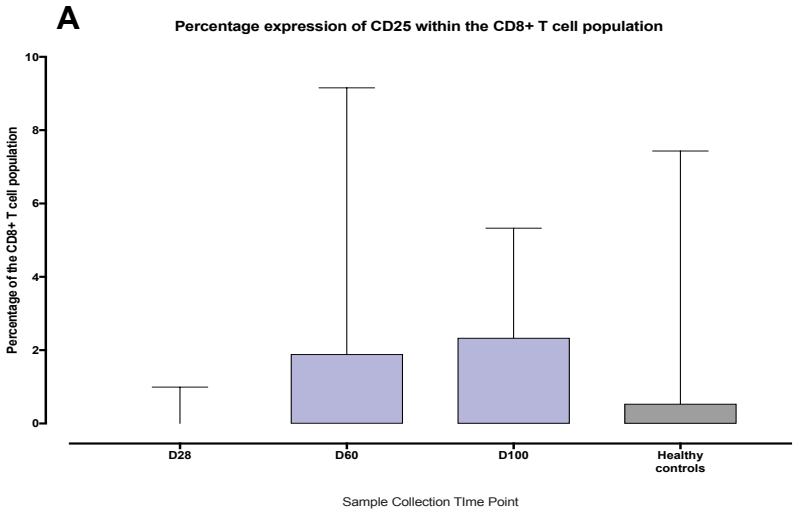
Additionally, the percentages of CD8+ T cells expressing activation markers (CD25, CD69 and HLA-DR) were measured in CBT patients.

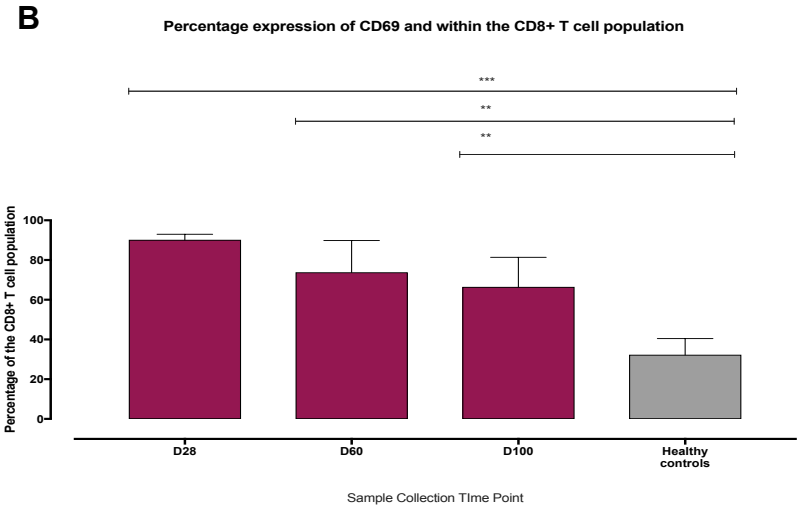
The median percentage of CD8+ T cells expressing CD25 (Figure 6.19A) was similar at day +28 (0%, range: 0-6%), +60 (2%, range: 0-14 %) and +100 (2%, range:0-9%) post-CBT compared to healthy controls (1%, range: 0-13%).

Furthermore, the median percentage of CD8+ T cells expressing CD69 (Figure 6.19B) was significantly higher at day +28 (90%, range: 43-100%), +60 (74%, range: 41-93 %) and +100 (66%, range: 32-86%) ($p=0.0003$, $p=0.002$ and $p=0.008$, respectively) post-CBT compared to healthy controls (32%, range: 30-45%).

Additionally, the median percentage of CD8+ T cells expressing HLA-DR (Figure 6.19C) was similar at day +28 (67%, range: 54-100%) post-CBT compared to healthy controls (64%, range: 41-68%). The median percentage of CD8+ T cells expressing HLA-DR was significantly higher at day +60 (96%, range: 86-99 %) and +100 (91%, range: 71-99%) post-CBT compared to healthy controls ($p=0.001$ and $p=0.0007$, respectively)

Overall, this data suggests that the expression of CD25 is equivalent to healthy controls in CD8+ T cells and there is a higher expression of CD69 and HLA-DR within CBT patients compared to healthy controls. Furthermore, this suggests that CD8+ T cells have mid and late activation within the first three months post-CBT.





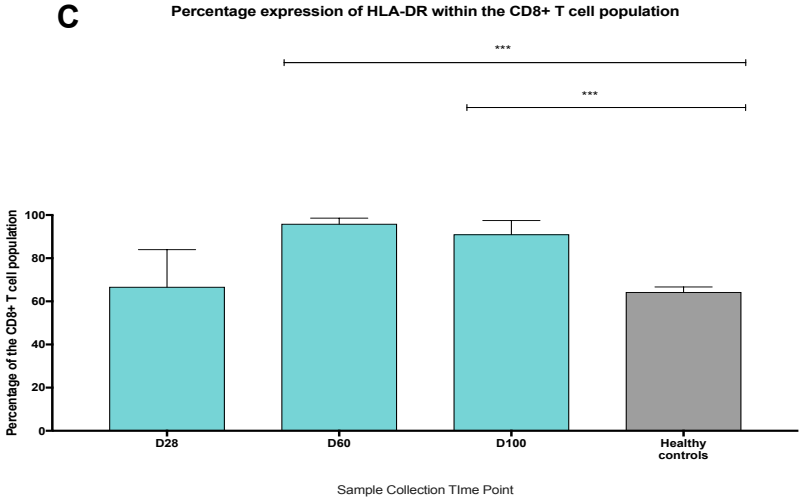


Figure 6.19 Percentage expression of activation markers (CD25, CD69 and HLA-DR) within the CD8+ T cell population of CBT patients. Box and whisker plots showing the percentage of CD25 (A), CD69 (B) and HLA-DR (C) activation markers within CD8+ T cells of CBT patients. At each time point the following number of samples were acquired: D28 = 14, D60 = 10, D100 = 10 and healthy controls = 5. Analysis was performed between the time points and the healthy controls. Analysis was performed between the time points and the healthy controls as a non-parametric, Mann-Whitney test. Results are presented as a tukey analysis. $p<0.01$ (**) and $p<0.001$ (***).

6.13 Discussion

In Chapter 3, it was shown that the there is delayed reconstitution of CD3+, and CD4+ T cells for up to 720 days post transplant. However, CD8+ T cell reconstitution takes place by 365 days. This corroborates data of other HSCT studies shown in Chapter 3, Table 3.2. In this study, TREC analysis was performed to assess thymic output. Furthermore, the kinetics of T cell reconstitution was measured to assess thymic and thymic independent reconstitution in CBT patients.

In this study, patients have a 1: 4 ratio of CD4:CD8 T cells at 28 days and between 60 and 365 days this shifts to a 2:1 ratio for CD4: CD8 T cells. However, by 720 days post-transplant there is a 1:1 ratio of CD4:CD8 T cells, which suggests that there is an increase in numbers of CD4+ T cells within the CD3+ T cell population. As previously shown, CD8+ T cells reconstitute by 365 days post-CBT, this could be the reason as to why there is a shift to a 1:1 ratio of CD4:CD8 T cells by 720 days. The increase in numbers of CD8+ T cells is also seen in other HSCT studies. In a study conducted by Alho *et al.* there is a 1:1 ratio of CD4:CD8 T cells in transplanted patients by 365 and 547 days post-transplant (Alho et al., 2016). Data within this project corroborates this, as there is a 1:1 ratio of CD4 to CD8 T cells in CBT patients within the same time frame. Furthermore, Komanduri *et al.* also demonstrates that there is an increase in the number of CD8+ T cells within the first year of transplant and in their cohort of patients there is an inverted ratio of CD4:CD8 T cells. This shows that there is an increase in absolute numbers of CD8+ T cells, post-transplant, compared to CD4+ T cells (Komanduri et al., 2007). Again, data within this current study verifies this data as we have showed that CD8+ T cells reconstitute sooner compared to CD4+ T cells.

To assess thymic output and thymic dependent reconstitution, the kinetics and diversity of naïve T cells reconstitution was measured. RTEs were identified via the detection of cell surface markers such as CD31. Furthermore, naïve T cells were identified through cell surface markers such as CD45RA and CCR7. In this project, the kinetics of reconstitution of naïve T cells (CD45RA+CCR7+)

was delayed in CBT patients for both CD4+ and CD8+ T cells. This corroborates a CBT study conducted by Komanduri *et al*. who demonstrated that were low absolute levels of CD45RA+CCR7+ naïve T cells in CBT patients for up to one year post-transplant (Komanduri et al., 2007). Respectively, the number of naïve T cells was also observed to be lower in dUCBT patients within a study conducted by Jacobson *et al.* for up to 18 months post-transplant (Jacobson et al., 2012). Data within this current study verifies this as CBT patients had low absolute levels of CD45RA+CCR7+ naïve T cells in PB post-transplant.

To further measure thymic output, the kinetics of reconstitution was measured by identifying the absolute numbers of RTEs in CBT patients. In healthy adults, CD31+ T cells are a distinct population of naïve T cells that are released from the thymus upon T cell maturation (Junge et al., 2007). CD31+ is a glycoprotein expressed on naive T cells that allows egression of T cells from the thymus (Kohler and Thiel, 2009). In this thesis, the absolute counts of CD31+ RTEs (CD4+ and CD8+) are lower compared to healthy controls and remain lower for up to 720 days post-transplant. This indicates that thymic output is reduced in CBT patients compared to healthy controls.

Moreover, CD31+ RTEs are known to contain high levels of TRECs (Gaballa et al., 2016, Kohler and Thiel, 2009, Ribeiro and Perelson, 2007, Sottini et al., 2014). In this thesis, TREC levels in CBT patients post-transplant are lower compared to healthy controls. Furthermore, TREC levels were undetected in the majority of patients for up to 720 days. In comparison, it has been shown that TREC levels are lower in CBT patients compared to BM and mPB recipients. Additionally, this has been highlighted in a study conducted by Komanduri *et al.* where absolute copy numbers of TRECs were lower compared to autologous and allogeneic grafts (Komanduri et al., 2007). Together, the data in this current study shows that there are low absolute counts of RTEs and low TRECs copy numbers. This could suggest that thymic output is reduced and delayed in CBT patients. Furthermore, this correlates with the fact there are low absolute levels of naïve T cells in CBT patients. Overall, this suggests that thymic output and thymic dependent reconstitution is delayed for up to 720 days in CBT patients.

Thymic output could be delayed in HSCT patients due to factors including the recipient's age, intensity of conditioning and whether GvHD develops. The recipient age is particularly important as thymic involution takes place in older individuals and this leads to a decrease in naïve T cell output (Lynch et al., 2009). Thymic activity is dependent on age and the ability for the thymus to recover is influenced by the recipient's age. The thymus is highly active during the initial six months of life and remains active throughout childhood. Over time, the thymus involutes and during this period the cellular cortical and medullary tissues reduce in terms of cellular content. Subsequently, this leads to a reduced proliferative expansion within the thymus and reduction in the release of early thymocytes (Douek et al., 1998, Haynes et al., 2000). Therefore, in CBT patients, thymic reconstitution may be prolonged, as lymphoid-myeloid progenitor cells will need to migrate to the thymus and undergo selection before maturing into a naïve T cell. However, there could be thymic involution in CBT patients, which impacts naïve T cell reconstitution post-transplant.

As previously described, the intensity of conditioning can also impact thymic recovery of T cells. An increase in intensity of conditioning can lead to a degree of thymic damage. Subsequently, this can impair negative selection of T cells within the thymus. This leads to the release of auto-reactive and allo-reactive T cells and ultimately leads to the development of GvHD. Delays in thymic output have been correlated with thymic damage in HSCT studies demonstrating that intense conditioning regimens impact and delay thymic recovery (Wu et al., 2013, Bacigalupo et al., 2009, Ballen et al., 2004, Corradini et al., 2005). Overall, it can be believed that intense conditioning regimens can induce thymic damage and this prolongs thymic recovery of T cells in CBT patients.

The development of GvHD can also impact thymic reconstitution in CBT patients. The development of aGvHD can delay thymic output and is correlated with a reduced number of naïve T cells in HSCT patients (MacMillan et al., 2009). This is due to the fact that the host lympho-haematopoietic system begins to serve as a target of allo-immunity and donor T cells. These T cells are alloreactive against recipient tissue antigens. GvHD is also known to interfere

with all stages of T cell development including: pre-thymic, thymic and post-thymic development (Aguila, 2010, Beschorner et al., 1978, Dulude et al., 1999). Furthermore, the administration of GvHD prophylaxis and immunosuppression can also delay naïve T cell output in CBT patients (Sabry et al., 2009). The morphology of the thymus can also change due to GvHD development, which causes reduction in the number of lymphoid cells and the composition of the stromal compartment of the thymus. This leads to a reduction in thymic output and it has been seen that aGvHD affects T cell maturation within the thymus. This occurs before rearrangement of the TCR β-chain gene and subsequently this leads to development of auto and alloreactive T cells in the thymus (Clave et al., 2009). Therefore, a balance is required post-transplant whereby the correct dose of GvHD prophylaxis is administered to patients to favour thymic development and suppress GvHD development. Unfortunately, in this current study it was not possible to make correlations between GvHD development and thymic reconstitution, as the GvHD data was not complete.

Aside from thymic reconstitution, thymic independent reconstitution of T cells was also measured in CBT patients. Thymic independent reconstitution is the transfer of mature/naïve T cells from the donor to the recipient. This leads to proliferation and expansion of T cells in the peripheral blood of the recipient. Additionally, naïve T cells can also differentiate into effector T cells (Mackall et al., 1993). The measurement and quantification of thymic independent T cell reconstitution was performed via the absolute counts of effector T cells, effector memory T cells and central memory T cells in the PB of CBT recipients. The presence of these T cells in healthy adults indicates the fundamental stages of T cell development that are required for adaptive immune responses. Herein, CD4+ effector T cells reconstitute in CBT patients by 60 days post-transplant. Furthermore, absolute levels of CD4+ effector T cells remain within the healthy control range for up to 720 days post-transplant. In contrast, the absolute counts of CD8+ effector T cells in CBT patients are lower compared to healthy controls and remain low up to 720 days post-transplant. This corroborates data by Komanduri *et al.* who also demonstrates that CD4+ effector T cells reconstitute within CBT patients between 30-60 days post-transplant

(Komanduri et al., 2007). A further allogeneic HSCT study by Alho *et al*. also demonstrates that there is an increase in the number of effector T cells in patients post-transplant and these patients experience homeostatic expansion of effector T cells post-transplant (Alho et al., 2016).

Effector T cells can differentiate and mature into two further subtypes of T cells known as effector memory and central memory T cells. These are two subsets of memory T cells with distinct homing potentials and effector functions of cell mediated immunity. Both effector memory and central memory T cells can respond to secondary stimulation of antigens and are crucial for the clearance of pathogens (Sallusto et al., 1999). Within this current project, CD4+ effector memory T cells reconstitute by 365 days and CD8+ effector memory T cells reconstitute by 100 days post-transplant. Komanduri *et al.* also shows this in CBT patients, where an increase in the number of CCR7- effector memory T cells is seen in CBT patients for up to one year post-transplant. Furthermore, the effector memory T cell population is the predominant population in the T cell compartment post-transplant (Komanduri et al., 2007).

Central memory T cells are CCR7+ and can migrate to lymphoid tissues where they are activated by antigens (Sallusto et al., 1999, Sallusto et al., 2004). Data from this current project demonstrates that the kinetics of CD4+ central memory T cell reconstitution is delayed in CBT patients and could take longer than 720 days to reconstitute. However, CD8+ central memory T cells reconstitute by 100 days post-transplant. As previously discussed, central memory T cells migrate to the lymphoid tissues and this could explain why there are fewer circulating central memory T cells present in the PB of CBT patients.

Thymic independent reconstitution could take place due to a number of reasons in CBT recipients. Early post-transplant, adoptively transferred donor T cells or recipient T cells that survive conditioning could proliferate, giving rise to increased numbers of effector memory and central memory T cells. The proliferation of the respective cells can also occur due to interactions with antigens (Mackall et al., 1996). Expansion of effector T cells in HSCT patients occurs from HPE, as the transfer of mature T cells replenishes the T cell

compartment. Furthermore, HPE takes place as adoptively transferred T cells undergo low affinity interactions with self-antigens (Goldrath and Bevan, 1999). This is further driven by cytokines such as IL-7, which is a non-redundant cytokine that stimulates naïve T cell expansion. IL-7 is known to regulate the naïve T cell pool and drives proliferation of naïve T cells in HPE (Mackall et al., 2001, Park et al., 2004). In CBT, it has been shown that IL-7 levels are elevated for prolonged periods of time and have been correlated to increase effector T cell and effector memory T cell numbers (Politikos et al., 2015). Future studies should measure plasma concentrations of IL-7 in CBT patients. The levels of IL-7 could then be correlated with the reconstitution of effector T cells in CBT patients. This could demonstrate whether there is an increase in effector T cell numbers due to increased IL-7 levels (Mackall et al., 1993, Politikos et al., 2015). Additionally, it has been determined that HPE occurs through the antigen dependent pathway where T cells circulating within the periphery undergo oilgoclonal expansion. This gives rise to increased numbers of effector memory and central memory T cells (Hakim et al., 1997).

T cell subsets were identified in this study to observe the composition of the CD4+ and CD8+ T cell compartments. By 720 days post-transplant, the CD4+ T cell population contained fewer naïve T cells and there was an increase in effector memory T cells compared to 28 days post-CBT. However, in the CD8+ T cell population there is a greater proportion of naïve T cells, effector memory T cells and central memory T cells. This corroborates data presented by Komanduri *et al*. who also showed similar proportions of the respective cells in the CD4+ and CD8+ T cell populations (Komanduri et al., 2007).

Furthermore, in HSCT, Tregs play a key role in mediating and suppressing immune reactions. In this current study, Tregs reconstitute within CBT patients by 365 days post-transplant. This verifies data by Kanda *et al*. who showed that Tregs reconstitution occurs by 365 days post-transplant in CBT patients. Treg reconstitution in CBT recipients has been compared to match sibling donors (MSD) and matched unrelated donors (MUD). The absolute counts of Tregs were similar in all three patient groups at 365 days post-transplant (Kanda et al., 2012). Additionally, increased absolute numbers of Tregs post-transplant

have been associated with improved immune reconstitution in haploidentical transplant recipients (Di Ianni et al., 2011).

In addition, all T cells express TCRs on the on the surface of the cell. It is known that 95% of T cells express αβ TCR and 5% of T cells express γδ TCRs. (Vantourout and Hayday, 2013). The expression of TCR αβ and γδ is poorly defined in CBT patients and therefore it was assessed within this study. Herein, 95% of CD4+ and CD8+ T cells expressed the αβ TCR. Respectively, 2% of T cells expressed the γδ TCR, this was similar to healthy controls. Therefore, the expression pattern of both TCRs in CBT patients is similar to healthy controls and this occurs by 28 days post-transplant. This suggests that there are no abnormalities in the reconstitution of the respective TCRs, in CBT patients.

To further understand the activation of T cells in CBT patients, the expression patterns of early, mid and late activation markers such as CD69, CD25 and HLA-DR, respectively, were measured. It was shown that CD4+ T cells highly express CD69 by 28 days post-CBT. Subsequently, the expression of CD69 decreased and there was an increase in the expression of CD25 and HLA-DR. This suggests that CD4+ T cells were activated early post-transplant. Similarly, the kinetics of activation markers, CD69 and HLA-DR, was the same in CD8+ T cells. However, a lower expression of CD25 was observed in CD8+ T cells. Overall, this demonstrates that the reconstituting CD8+ T cells were activated early post-transplant. The activation of CD4+ and CD8+ T cells could be due to the pro-inflammatory environment within CBT patients, within the first three months post-transplant. This could induce the activation of T cells and therefore increase the expression of the respective activation markers.

In CBT recipients, it is known that T cell reconstitution is delayed due to various factors. Host-related factors such as age can influence immune reconstitution as thymic involution limits the output of naïve T cells. Also, graft related factors could also affect the reconstitution of T cells post-transplant. It has been demonstrated that low doses of TNCs and T cells in the graft can impact thymic independent recovery in CBT patients (Niehues et al., 2001, Douek et al., 1998, Mackall et al., 1995). HLA disparity can also be considered as a graft related

factor that impacts T cell recovery. In CBT, there are fewer matching requirements between the donor and the recipient compared to other HSCT. This could mean that patients could have a higher degree of mismatches between the donor and the recipient, which could impact thymic selection and output and lead to a skewed TCR repertoire (Roux et al., 2000).

Additionally, treatment related factors such as pre-transplant conditioning or the transplant procedure could also impact T cell reconstitution. T cell depleting agents such as ATG are commonly used post-transplant and can impact thymic independent reconstitution of T cells (Admiraal et al., 2015, Lindemans et al., 2014). However, in this current study no patients were given ATG. Moreover, the use of myeloablative conditioning regimens and radiation-based regimens could also cause damage to the thymus. This could mean that the thymus would need to repair before it can resume T cell output to healthy control levels (Chung et al., 2001). In this current study, 17 patients had a MAC transplant.

Another factor that can impact T cell reconstitution is the development of GvHD. This can impact thymic reconstitution due to increased numbers of alloreactive T cells within the HSCT recipient. Alloreactive T cells can damage stromal and lymphoid compartments of the thymus, where T cell development and selection takes place. Thymic damage induced by GvHD can delay the output of naïve T cells from the thymus (Krenger and Hollander, 2008, Clave et al., 2009, Uhlin et al., 2012). Additionally, GvHD prophylaxis is administered to patients to suppress inflammatory responses and prevent GvHD development. Corticosteroids as part of GvHD prophylaxis are potent lympho-depleting agents, which could reduce T cells within the CBT recipient. This therefore causes a delay in T cell reconstitution.

As far as we are aware, this is the only study in the UK that extensively measures the kinetics and diversity of T cell reconstitution in CBT patients. Overall, data in this study shows that T cell reconstitution is delayed in CBT patients and specifically thymic-dependent reconstitution is severely delayed. Furthermore, there is an increased absolute count of effector memory and

central memory T cells in CBT patients, which occurs by 365 days post-transplant.

Future studies and improvements should be undertaken to improve thymic output and to decrease the delay in naïve T cell reconstitution in CBT patients. The expansion of central memory and effector memory T cells post-transplant could also suggest an increase in T cells that can target towards infection or a balanced GvL effect. However, infection data was not complete and correlations could not be made between infection and the delay in immune reconstitution of T cells. To further understand the diversity of T cell receptors, TCR spectratyping could have provided a further insight into the restoration of TCR clones in CBT patients. This could have demonstrated the TCR diversity and whether reconstituting T cells can identify a variety of antigens.

To better understand thymic independent expansion of T cells, future immune reconstitution studies in CBT require culturing patient T cells with IL-7 and measuring the proliferation via the use of CFSE and KI67. This would aid in determining whether T cells can proliferate in response to cytokine stimulation and their proliferative capacity can be measured through CSFE staining. Further functional studies are also required to understand whether reconstituting T cells in CBT patients aid in the clearance of infection and whether they partake in GvL responses in CBT patients.

Chapter 7 : Discussion and future work

7.1 Background of the current study

Reconstitution of immune cell subsets and balanced immune function is essential in CBT recipients, as it will reduce the development of post-transplant complications such as relapse, GvHD and infection. Immune reconstitution in HSCT has been studied internationally. However, a comprehensive study of immune reconstitution in CBT patients has not been performed in the UK. Studying immune reconstitution in CBT patients will provide an insight into the kinetics of recovery of the immune cell subsets in CBT patients. Hence, the aim of this study was to investigate the kinetics and diversity of reconstituting immune cell subsets in CBT patients within the UK. This chapter summarises and discusses data from this project and its relevance to clinical practice and proposes potential future work to extend the project.

7.2 Physiological relevance of this study

7.2.1 Haematopoietic recovery and engraftment

Immune reconstitution is an important facet of CBT. Delayed or defective immune reconstitution can lead to post-transplant complications such as GvHD, relapse and infection in CBT patients. One of the major problems of using CB as a source of HSC is the delay in immune reconstitution in comparison to PB and BM graft sources (Komanduri et al., 2007, Ruggeri et al., 2011). Haematopoietic recovery is an important measure of immune recovery in CBT and is clinically measured through the use of TNC counts, neutrophil engraftment and platelet engraftment (Kim et al., 2014). Neutrophil engraftment is clinically recorded as the first three consecutive days of neutrophil counts greater than 0.5 $x10^9$/L. Neutrophil engraftment occurs in the majority of CBT patients and can take place between 5-21 days (Laughlin et al., 2004, Rocha et al., 2004, Seggewiss and Einsele, 2010). Respectively, platelet engraftment is clinically recorded as three consecutive days of platelet counts at greater than $20x10^9$/L. The median time to platelet engraftment varies in CBT studies and occurs between 50 and 100 days post-CBT (Petropoulou and Rocha, 2011). Comparatively, this current study shows that the recovery of neutrophils and platelets occur within a median time of 20 days and 40 days, respectively. The time to engraftment can differ between patients and this is due to a number of factors including: the severity of the underlying disease; preparative conditioning used to clear the residual disease and pre-transplant radiotherapies and chemotherapies. Furthermore, TNC and CD34+ counts are an important factor for efficient engraftment in CBT patients. It has been shown that increased CD34+ cells and higher TNCs are associated with an increased probability and rate of neutrophil and platelet engraftment (Purtill et al., 2014, Danby and Rocha, 2014, Gluckman et al., 2004). It has also been reported that patients who receive G-CSF in the post-transplant period have a better rate of engraftment (Konuma et al., 2015). This highlights that there are number of clinical practices affecting the time in which engraftment takes place between patients.

A broad understanding of leucocyte reconstitution can be achieved through the measurement of CD45 expression. CD45 is a known as the common leucocyte antigen and has been used in this study to measure the kinetics of leucocyte reconstitution. As previously described, leucocyte reconstitution is clinically measured through neutrophil and platelet engraftment, which is validated on many automated analysers. However, in this study an automated analyser was not available. Therefore, CD45 was used to identify the expression patterns of leucocytes post-transplant and it has been shown that CD45+ leucocyte recovery takes place by 60 days post-transplant.

7.2.2 Monocytes in cord blood transplant

The reconstitution of CD14+ monocytes is poorly defined in CBT patients. However, in a recent study by Li *et al.* the reconstitution of CD14+ monocytes in CBT patients occurs by seven days post-transplant. Furthermore, the absolute counts of CD14+ monocytes were higher than the healthy control range in CBT patients for up to five years post-transplant (Li et al., 2017). Herein, it has been shown that there is early reconstitution of CD14+ monocytes, which occurs by 28 days post-transplant. The exact function of monocytes in CBT patients is currently unexplored and the early reconstitution of monocytes highlights that their function should be investigated.

In healthy adults, monocytes are able to prime naïve T cells and induce T cell proliferation (Bhardwaj et al., 1994). Additionally, monocytes play an active role in inflammation and upon antigenic stimulation, they can release pro-inflammatory cytokines such as TNF-α and IL-1β (Ziegler-Heitbrock, 2007). This shows that monocytes have an immunomodulatory and pro-inflammatory role in healthy adults.

In contrast, HSCT patients can be treated with corticosteroids post-transplant and there is an increase in the number of monocytes in patients treated with prednisolone. Furthermore, monocyte absolute numbers increase in patients with aGvHD. This causes further recruitment of Th17 cells and exacerbation of inflammatory responses. Therefore in HSCT, monocytes could recruit

inflammatory cells to induce GvHD responses (Reinhardt-Heller et al., 2017). Additionally, it is known that monocytes release BAFF upon activation. BAFF is a cytokine that promotes survival of B cells (Batten et al., 2000, Rolink et al., 2002). This could explain why there are increased absolute numbers of B cells in CBT patients. However, future work is required to measure BAFF release from monocytes in CBT patients. This is highlighted later in this chapter.

With the various functions of monocytes in mind, future work could be undertaken to further identify subpopulations of monocytes in thawed patient samples. This could be performed by identifying subpopulations of monocytes in CBT patients via the expression patterns of CD14 and CD16 using flow cytometry. These are both typical markers used to define monocytic populations (Rogacev et al., 2015). However, little is known about the number of monocytic cells in CBT recipients and their function post-transplant. Further efforts are required to elucidate their reconstitution patterns and role post-transplant.

7.2.3 NK cell function in cord blood transplant

NK cell reconstitution occurs within the first month post-CBT (Brahmi et al., 2001, Jacobson et al., 2012, Ruggeri et al., 2011, Small et al., 1999, Somers et al., 2013, Thomson et al., 2000). Early, post-CBT, there are higher percentages of immature $CD56^{bright}$ NK cells. These NK cells then acquire the $CD56^{dim}$ phenotype within the first three months post-transplant (Cooley et al., 2005, Nguyen et al., 2008). $CD56^{dim}$ NK cells have a significantly higher cytotoxicity compared to $CD56^{bright}$ NK cells (Cooper et al., 2001, Nagler et al., 1989). In this thesis, a higher proportion of $CD56^{dim}CD16^{negative}$ NK cells were present in CBT patients post-transplant compared to $CD56^{bright}$ $CD16^{negative}$ and $CD56^{dim}$ $CD16^{postive}$ NK cells. It is possible that these NK cells had a functional response to clear infection or could have been apart GvL related responses. However, further work is required to elucidate their functions post-transplant and this is described later in this chapter. Additionally, there is limited data on the function of $CD56^{dim}CD16^{negative}$ NK cells in healthy adults and HSCT recipients. Poli *et al.* also highlights this that this subpopulation is present within healthy individuals. However, little is known about the function of this subpopulation of NK cells

(Poli et al., 2009). This highlights that further studies are required to elucidate the functions of the $CD56^{dim}CD16^{negative}$ NK cell subpopulation post-CBT.

Post-transplant, the immune system in CBT patients is highly inflammatory. NK cells become activated through the interaction with various antigens leading to the upregulation of stimulatory markers such as CD69 and HLA-DR (Pittari et al., 2010). Subsequently, this leads to the induction of cytotoxicity of the target cell via NK cells (Moretta et al., 1991). Early expression of both CD69 and HLA-DR in CBT patients shows that early-reconstituted NK cells are prone to activation (Bjorkstrom et al., 2010). In this thesis, NK cells are activated which has been demonstrated by high expression of CD69 and HLA-DR.

Activation and inhibitory markers such as NKG2A and NKG2C can also define the functionality of NK cells in CBT patients. Beziat *et al.* has shown that NK cells express NKG2A, an inhibitory receptor, which is expressed for up to three months post-transplant. Subsequently, the expression of NKG2A decreases and there is an increase in the expression of NKG2C, an activating NK cell receptor. This demonstrates that NK cells are activated post-transplant and there is a shift from inhibitory NK cells to activated NK cells after three months post-transplant (Beziat et al., 2012, Beziat et al., 2011, Beziat et al., 2009). CD57 further defines the maturation of NK cells in CBT patients. NK cells can be defined as CD57+NKG2A+ or CD57+NKG2C+, which are inhibitory-mature or activating-mature, respectively. In HSCT, the kinetics of reconstitution of inhibitory-mature NK cells occurs in the first three months post-transplant. This then shifts to an activating-mature NK phenotype after several months (Bjorkstrom et al., 2010, Lopez-Verges et al., 2010). Herein, there was a higher proportion of inhibitory-mature NK cells (NKG2A+CD57+) in CBT patients. However, this was investigated in the first 100 days post-CBT and further observations of the respective phenotypes could not be made due to limited patient samples at later time points. Additionally, Beziat *et al.* also demonstrates that there is rapid maturation of NK cells post-transplant that express activating receptors, NKp30 and NKp46, which are believed to indirectly override the NKG2A-inhibitory signal to allow elimination of leukaemic cells (Beziat et al., 2009). Future work to further elucidate the function of NK cells post-transplant

would include determination of activating receptors such as NKp30, NKp46, and NKp80 via flow cytometry. The patterns of expression of activation markers could then be correlated with the expression patterns of NKG2A/NKG2C. This would aid in identifying the actions of NK cell cytotoxicity.

NK cell function can also be defined by the expression of cytokines such as IFN-γ. IFN-γ is an important inflammatory cytokine that can be produced by NK cells. Cytokines such as IL-12 and IL-18 secreted by macrophages and DCs can induce the production of IFN-γ in NK cells (Matikainen et al., 2001). Commonly post-transplant, patients are prone to infections and IFN-α is produced during viral infections. This synergises with IL-12 and IL-18 to induce the production of IFN-γ. Respectively, TNF-α is also produced and induces the production of IFN-γ (Marshall et al., 2006). It has been shown that NK cells in CBT and allogeneic HSCT recipients produce IFN-γ, which could be released to clear infections, induce GvL responses and clear residual disease (Beziat et al., 2009, Smyth et al., 2002). In this current study, the intracellular expression of IFN-γ in unstimulated isolated NK cells in CBT patients is high. This suggests that NK cells are stimulated post-transplant and possess the capacity to express IFN-γ. Given that the immune environment is highly pro-inflammatory post-transplant, the production of IFN-γ could be required to induce cytotoxic responses of NK cells. With this in mind, one could hypothesise that the expression and release of IFN-γ from NK cells in CBT patients could aid in immune responses to clear pathogens or drive NK cell cytotoxicity directed to residual disease. To test this hypothesis, *in vitro* killing assays could be performed. Isolated NK cells from CBT patients, stimulated with IFN- γ, would be used to test this. These NK cells would then be tested in an *in vitro* killing assay to assess their cytotoxic capacity against leukaemic cell lines such as K562, Daudi and Raji as well as primary AML blasts from patients. Respectively, the results would be compared to healthy controls and unstimulated NK cells from CBT patients.

The cytotoxic capacity of NK cells is a key functional role that allows NK cells to directly lyse target cells. In HSCT, this is important as NK cells could be involved in clearing residual disease or clearing infection. NK cells in CBT

patients have been shown to directly lyse K562 target cells and AML blasts. Furthermore, the cytotoxic capacity of CBT recipient NK cells is equal to healthy adults. However, the cytotoxic capacity of CBT recipient NK cells is higher compared haploidentical HSCT recipient NK cells (Nguyen et al., 2005, Beziat et al., 2009, Nguyen et al., 2009). As T cell reconstitution is delayed in CBT patients, NK cells could play a crucial role in GvL responses. Additionally, NK cells switch from an immature to mature phenotype within the first-year post-transplant and are stimulated and activated with a functional capacity to lyse target cells.

With the entire functions of NK cells in mind, further work can be undertaken to better define their role in CBT recipients. In this thesis, three main NK cell subsets have been identified within CBT patients. Future work includes the isolation of $CD56^{bright}$ $CD16^{negative}$, $CD56^{bright}$ $CD16^{positive}$ and $CD56^{dim}CD16^{positive}$ and $CD56^{dim}CD16^{negative}$ NK cells from patient samples. The respective subpopulations could then be co-cultured with leukaemic blast cells and their degranulation could be tested through the identification of CD107a. This would be measured via flow cytometry. Additionally, isolated NK cells could be cultured with leukaemic cell lines such as Daudi, Raji, RPMI8226, U266 and U937. This would demonstrate whether NK cells possess a cytolytic capacity against a broad range of leukaemic cells. Furthermore, the use of primary cell blasts from AML patients could also be used to better understand whether isolated NK cells from CBT patients can directly lyse leukaemic cells within patients. ADCC cytotoxicity can also be measured in future work where NK cells isolated from CBT patients could be directed against Raji cells and the degranulation of CD107a can be measured to assess the level of cytolytic activity (Shabrish et al., 2016).

7.2.4 B cells in cord blood transplant

B cells are the second lymphocyte cell type to reconstitute in CBT patients after NK cells. KREC analysis and absolute counts of B cells have shown that B cell reconstitution takes place between three and six months post-CBT (Nakatani et al., 2014). In this current study, B cell reconstitution takes place in CBT patients within 100 days post-transplant, which has been shown via KREC analysis and absolute count measurement of CD19+ B cells.

In allogeneic transplant, transitional B cells are the first subtype of B cell to enter the periphery from the bone marrow. In HSCT, absolute numbers of transitional B cells are higher in CBT patients compared to BMT recipients (Nakatani et al., 2014). At later time points, such as one year post-transplant, the absolute counts of transitional B cells decrease and there is an increase in the absolute number of mature B cells (Seggewiss and Einsele, 2010, Marie-Cardine et al., 2008). Data within this thesis shows that the majority of B cells in CBT patients are transitional B cells and by 365 days post-CBT there is an increase in the absolute number of mature B cells. In CBT studies, the majority of B cells in patients are of a transitional phenotype and these studies have shown that the functionality of B cells is also restored in patients within the first-year post-transplant (Charrier et al., 2013, Servais et al., 2014).

In this current study, there are higher absolute numbers of B cells in CBT patients compared to healthy controls after 100 days post-transplant. In HSCT studies comparing mPB and CBT, an increase in the absolute number of B cells was associated with an increase in the cytokine BAFF. This cytokine is known to promote the survival of B cells (Jacobson et al., 2012). It could be released by B cells and could enhance the survival of B cells in CBT patients. Furthermore, the secretion of BAFF could lead to the increased proliferation of B cells post-transplant. Additionally, monocyte reconstitution is rapid and exceeds the number of monocytes seen within healthy adults. Activated monocytes can produce BAFF, which could stimulate the survival of B cells (Batten et al., 2000, Rolink et al., 2002). Therefore, increased numbers of B

cells post-transplant could be maintained through the presence of activated monocytes. In this thesis, the absolute number of CD14+ monocytes could be associated with the increased absolute numbers of B cells post-transplant. However, to elucidate this, measurement of BAFF in the serum of patient samples can be measured. This could be performed using an ELISA assay. The measurements could then be correlated to B cell reconstitution. Furthermore, monocytic cells could be isolated and their expression patterns of BAFF could be measured to further understand whether they possess the capacity to express and release BAFF.

Aside from B cell reconstitution, B cell function has been demonstrated to also occur within the first year post-transplant. Within the first year post-transplant, there were higher numbers of naïve and transitional B cells in CBT recipients. However, the increase in mature B cells demonstrates that maturation was taking place within CBT patients. CBT studies have shown that there are higher levels of IgG in CBT patients compared to mPB and BM recipients (Jacobson et al., 2012). To better understand the reconstitution of B cell functionality, immunoglobulin measurements should be measured in CBT patients. Moreover, future work would include the measurement of IgG and IgM in CBT patient serum samples, via ELISA assays. Additionally, serum electrophoresis can be performed to test for monoclonal, oilgoclonal and polyclonal immunoglobulins. This would provide a greater understanding of B cell functionality and the maturation of B cells in CBT patients. Additionally, this would also identify the antibodies produced by B cells post-transplant. This could be correlated to B cell reconstitution in CBT patients and depending on the serum Igs present, correlations to functional maturity could be made. BCR spectratyping can also be undertaken as future work, which could be used to determine the broadness of the BCR repertoire in different groups of B cells (Wu et al., 2015).

7.2.5 T cells in cord blood transplant

Delayed T cell reconstitution still remains a challenge in CBT. In a study conducted by Komanduri *et al.* it has been shown that T cells are the last lymphocytes to reconstitute within CBT patients and they recover between one and two years post-transplant (Komanduri et al., 2007). The kinetics of T cell reconstitution in this current study shows that complete T cell recovery could take longer than two years to recover.

In CBT, thymic recovery is specifically delayed (Kanda et al., 2012, Komanduri et al., 2007, Ruggeri et al., 2011, Servais et al., 2014). CBUs contain 10-100 fold fewer HSC and progenitors cells compared to BM and mPB grafts and this can result in delayed thymopoeisis. Therefore, in dUCBT patients, the complete absence of TRECs demonstrates that there is a delay in progenitor cells homing to the thymus and reconstituting the naïve T cell population. Furthermore, the absence of TRECs could also be due to a lack in thymic function (Komanduri et al., 2007). Data within this thesis shows that there are low absolute levels of RTEs post-transplant, which validates that there is delayed thymic recovery in CBT patients. Thymic reconstitution is affected by a patient's age and younger individuals show to have better thymic recovery compared to older ones. This is due to thymic involution in older patients, which causes a decrease in thymic output (Lynch et al., 2009). The median age of CBT recipients in this current study is 48 years old and thymic involution in these patients could contribute to the delay in thymic output post-transplant.

In CBT recipients, T cell reconstitution takes place through HPE and the majority of engrafting T cells are naïve and antigen inexperienced. A faster increase in peripheral T cell numbers are seen in recipients of dCBUs compared to sCBUs. Furthermore, increased HLA-disparity between the CBUs and the recipient could induce expansion of T cells through graft-versus-graft interactions (Lamers et al., 2016). It has been shown that fetal T cells convert rapidly compared to adult T cells into memory T cells upon proliferation (Early and Reen, 1999). This therefore increases the skewing towards an effector and central memory phenotype of T cells in CBT patients (Komanduri et al., 2007).

Data from this current study shows that there is a high proportion effector memory T cells in CBT recipients by 720 days post-transplant. This could suggests that there is either HPE of T cells in CBT patients or naïve T cells from the graft that have differentiated into effector memory T cells.

Tregs are a subpopulation of T cells that can suppress immune responses and have immunoregulatory roles. In CBT recipients, Tregs reconstitute within the first year post-transplant. However, absolute counts of Tregs remain low within the first six months post-CBT (Jacobson et al., 2012). In this current project, Treg reconstitution occurs by 365 days post-transplant. The delay in reconstitution of Tregs could increase the chances of aGvHD and cGvHD development in CBT patients. Future efforts must be undertaken to improve the kinetics and reconstitution of Tregs in CBT patients and current on-going studies explore the use of Treg therapy, discussed later in this chapter. The aim of using Treg therapy in CBT patients is to suppress GvHD related reactions post-transplant.

Overall, T cell reconstitution is delayed in CBT recipients post-transplant. Additionally, CBT patients could have reduced TCR diversity post-transplant. Both T cell diversity and TCR repertoire diversity are key elements in the recovery of immune competence. Defects in the reconstitution of a broad T cell diversity may limit the T cell-based GvL effect and predispose the patient to relapse (Talvensaari et al., 2002). It has been demonstrated that CB T cells lose their naïve phenotype in an antigen independent manner and at the same time antigen-driven proliferation of CB T cells causes oligoclonal expansion and reduction in the TCR diversity (Li and Xu, 2015). With this in mind, TCR spectratyping can be performed through future work to distinguish the TCR repertoire diversity in CBT patients. This could provide a global analysis of the distribution and clonal expansion of TCR subfamilies in CBT patients

7.3 Caveats and weakness of the study

This study presents the results of kinetics and diversity of reconstituting cell subsets in CBT recipients. However, there was an immunophenotyping limitation where a four-colour immunofluorescent flow cytometry panel was set up in 2009 during the initial study design. This only allowed four surface markers to be identified on cell subsets at any given time. An increased number of surface markers could have been identified if more immunofluorescent colours could have been detected.

Moreover, the reduction in the number of patient samples at later time-points was an additional limitation of this study. Samples could not be collected at certain time points due to poor health of the patients. The reduction in patient samples meant that there was limited data collected at later time points and this impacted the analysis undertaken to compare across and between time-points and healthy controls.

Additionally, the retrieval of clinical data respective to the patient was also a limitation in this current study. In particular, infection, GvHD and relapse data were not complete and data was not available from transplant centres. This prevented correlations being made with reconstituting subsets. If future CBT or HSCT studies are performed then clinical data should be recorded to the highest level.

The NK cell functional assays were performed on thawed PBMCs and thawed isolated NK cells. Limitations of this included a decrease in the viable number of PBMCs, which subsequently decreased the number of viable NK cells collected after isolation. Due to this, the number of patient samples analysed decreased as a viable NK cell population above 90% was required to perform cytolytic functional studies, as described in Chapter 2, Section 2.8.6.

Additionally, due to the low absolute numbers of T cells within the patient samples, T cell functional studies could not be performed. This limited further understanding of T cell functionality in CBT patients.

An additional caveat of this study was that CD107a phenotyping could not be performed to identify NK cell degranulation. The isolated NK cells were not able to withstand the cell staining procedures and a high level of cell death was seen in the tested samples. Moreover, due to limited patient sample numbers, further tests of NK cell cytotoxicity directed against other leukaemic cell lines and patient leukaemia cells could not be performed. This could have demonstrated that NK cells in CBT patients have a broad range of cytotoxicity directed to various leukaemic cell lines. In addition, further NK cell functional studies against patient leukaemic cells could demonstrate whether reconstituting NK cells possess the capacity to lyse patient malignancies. This could have demonstrated whether CBT recipient NK cells play a vital role in GvL responses. With this in mind, future NK cell functional studies should be performed on freshly isolated NK cells to avoid the described issues.

Moreover, in this study the reconstitution of B cells was shown. However, functional studies were not performed, as the viability of B cells recovered post-thawing was very low. Future analysis of B cells and the impact on clinical outcomes in CBT is required, as B cells are one of the first major lymphocytic populations to reconstitute post-transplant. Additionally, a cytokine release analysis could have been performed to better understand the secretion patterns of pro-inflammatory and anti-inflammatory cytokines. The detection of IgM and IgD could have been analysed through ELISA assays. The incorporation of these tests could have given a better insight into the functionality of B cells. Future studies should include these tests when understanding B cell function in CBT patients as it will enhance the understanding of the function of B cells post-transplant.

7.4 Translation to the clinic

This study is the first within the UK to measure the kinetics and diversity of reconstituting immune cell subsets in CBT patients. It also serves as a clinical timeline: (1) for clinicians and scientist to have a better understanding of the kinetics and diversity of reconstituting immune cells in CBT patients and (2) to provide information for clinicians and research staff to investigate how to improve reconstitution of immune cell subsets in CBT patients. It is hoped if we have a better understanding of immune reconstitution we may be able to improve outcomes post-transplant.

In this sample of UK performed CBT, the median time to neutrophil and platelet engraftment in CBT patients within the UK is 20 and 40 days, respectively. Various methods described in Chapter 1, Section 1.10.6, Table 1.2 are being undertaken to improve the time to engraftment in CBT patients.

Through this study, it was shown that monocytes, NK cells and B cells are the first three lymphocytic populations to reconstitute in CBT patients. This occurs within the first three months post-transplant. NK cells are known to reconstitute rapidly, irrespective of the graft source and they play a key role in mediating GvL. Currently, there are various clinical trials that are on-going to understand the functions of NK cells in CBT patients (clinicaltrials.gov, Trial Numbers: NCT01619761, NCT02727803). The specific aims of these studies are to identify whether the infusion of NK cells post-transplant can reduce the incidence of relapse and decrease the risk of GvHD. Furthermore, the aim of these trials is to potentiate donor NK cell killing of cancer cells by *ex vivo* expansion. This will deliver various functions of NK cells where the reconstitution of NK cells have the potency to become alloreactive as demonstrated by the KIR-KIR ligand incompatibility hypothesis (Ruggeri et al., 2005). Additionally, the infusion of NK cells post-transplant could be used to reduce the incidence of GvHD by inhibiting donor alloreactive T cells (Olson et al., 2010).

As previously described, B cell reconstitution is rapid in CBT patients and occurs within three months post-transplant. The increased absolute counts of B cells early post-transplant have been demonstrated to reduce the incidence of cGVHD (Jacobson et al., 2012). The main future directions of understanding B cell function in CBT patients is being investigated to elucidate their role in GvHD. Early reconstitution of B cells could indicate that they play a role in GvHD reactions. Various studies are investigating the effects of B cell depletion and the incidence of GvHD post-transplant, particularly demonstrated through the use of Rituximab (Bhatt et al., 2016).

T cell reconstitution is severely delayed in CBT patients and it is fundamental for T cells to reconstitute to improve outcomes such as relapse or to prevent/treat infection. There has been much effort to use adoptively transferred T cells to improve immune reconstitution. This enhances the time in which infection can be treated or prevented in CBT patients. However, in the context of CBT, *ex vivo* strategies have been explored using Dynabeads, IL-2 and anti-CD3/CD28 (Mazur et al., 2008, Okas et al., 2010). The expansion of T cells was possible although there was a high level of apoptosis of CD4+ T cells. Furthermore, IL-7 was used to reduce apoptosis and this promoted functional maturation of T cells. These expanded T cells lacked all reactivity against allogeneic cells but they could be primed against leukaemic cells (Davis et al., 2010). Further clinical trials of expanded T cells are necessary, which would provide an understanding of T cell function post transplant and whether infused T cells can aid in the clearance of residual disease and clearance of infection.

Moreover, further work has been undertaken to understand whether pathogen specific T cells can be expanded and primed against CMV antigens. This was undertaken through the isolation of CB T cells that were primed with IL-7 and IL-12. These T cells were then stimulated with monocytes and DCs containing a CMV antigen (Hanley et al., 2012). This demonstrates a potential for *ex vivo* T cell priming for the clearance of specific pathogens. Additionally, cytotoxic T cells have been engineered via GMP methods that are directed to CMV, EBV and Adenovirus (ADV). These T cells have been stimulated via CB-DCs that have been transduced with ADV vector containing a CMV antigen. The

cytotoxic T cells are cultured in IL-7, IL-12 and IL-15. Thereafter, a second stimulation is undertaken using EBV transformed B cells. The results from this study are promising and only 25% of the CBU is used to produce in excess of $200x10^8$ viral specific cells (clinicaltrials.gov: trial number: NCT00880789). This highlights the potential for CB T cells to be primed and stimulated against pathogens and could be infused into patients with the hope of clearing opportunistic infections post-transplant.

Tregs play a crucial role in transplantation tolerance within HSCT patients. Current therapeutic strategies are used to expand Tregs for infusion into HSCT patients to suppress immune system activation and promote immunologic tolerance. Tregs can modulate innate and adaptive immune responses and are used to improve outcomes post-transplant. Adoptive transfer of Tregs has been demonstrated as a potential immunotherapeutic strategy (June and Blazar, 2006). Banked CBUs are used as a source of Tregs, which are isolated and expanded via stimulation with anti-CD3/CD28 microbeads and then stimulated with IL-2. The expanded Tregs have polyclonal reactivity due to non-specific TCR stimulation. Current on-going studies (clinicaltrials.gov trial number: NCT02991898, NCT02423915, NCT01937468) are now testing the infusion of these specifically expanded Tregs to better understand the dose of administration but also to review the outcomes of patients infused with these Tregs. Furthermore, the aim of these studies is to address whether the infusion of Tregs suppresses or reduces the incidence of aGvHD.

Taken together, data within this study provides a better understanding of immune reconstitution in CBT patients within the UK. All of the findings from this current project can facilitate decision making for future clinical applications of CBT and post-transplant therapeutics. Furthermore, findings from this study highlight key areas that can be improved to enhance immune reconstitution in CBT patients.

Chapter 8 : Appendix

8.1 Poster Presentations

- UCL Infection and Immunology Symposium, 2014
- UCL Infection and Immunology Symposium, 2015
- BSI Oxford Immunology meeting, 2016
- EBMT - Marseille, 2017

8.2 Publications

Feliu J, Clay J, Raj K, Barber L, Devlia V, Shaw B et al.
Transplant-acquired food allergy (TAFA) following cord blood stem cell transplantation in two adult patients with haematological malignancies. Br J Haematol. 2014 Nov;167 (3): 426-8

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